University of Alberta

Neurochemical and Neuroprotective Properties of Phenylethylidenehydrazine Analogues

by

Bernard Sowa ©

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DEDICATION

To my supervisor, Dr. Glen Baker, I wish to express my eternal gratitude for the opportunities with which you have provided me, for your gentle guidance and your trust. To my co-supervisor, Dr. Kathryn Todd, I thank you for your confidence in my abilities, your encouraging words, your guidance and advice. To my wife, Efosa, your love, gentle spirit, support and patience have been a fountain of strength to this "professional student" and I thank you for your unwavering support. To my parents, Bernard and Davida, your love, wisdom, morals, integrity and beliefs provided the base upon which I stand today. To my sister, Davida, your achievements, continued faith in me and support for my decisions have served to motivate me more than I have ever spoken. Finally, I thank God, without whom none of this would have been possible and because of whom all of this has come to be.

ABSTRACT

Studies were carried out with the goal of determining the neurochemical properties and neuroprotective potential of several novel γ-aminobutyric acid (GABA)-elevating agents derived from phenylethylidenehydrazine (PEH). Three agents, 4-fluoro-PEH (FPEH), 4-methyl-PEH (MPEH) and 4-methoxy-PEH (MeOPEH) were examined with respect to their effects (acute and chronic) on gerbil brain amino acid levels, GABA-transaminase (GABA-T), alanine-transaminase (ala-T), monoamine oxidase (MAO), cytochrome P450 (CYP) enzyme-catalyzed metabolism and core temperature as well as their neuroprotective efficacies in gerbil hippocampus (transient forebrain ischemia) and rat hippocampus (DSP-4 toxicity). Acute administration of the PEH analogues was associated with significant inhibition of brain GABA-T and ala-T, increases in brain GABA and alanine levels and transient reduction of core temperature. Phenelzine (PLZ), which inhibits monoamine oxidase (MAO) in addition to GABA-T, caused a more marked reduction in body temperature in the gerbils than did PEH or its three analogues. FPEH and PEH were further examined for their effects on GABA-T and ala-T *in vitro* and caused dose-dependent inhibition. FPEH was also studied for its effects on MAO-A and -B *ex vivo* and, in contrast to PLZ, produced only weak inhiibition of MAO. FPEH, in contrast to MePEH and MeOPEH, caused a decrease in brain levels of glutamate (acutely and chronically) and glutamine (acutely). All of the analogues interfered with CYP-mediated metabolism, with the effects generally being more potent on CYPs 1A2, 2B6 and 2C19 than on 2D6 and 3A4.

Despite having similar effects on the enzymes GABA-T and ala-T as well as on brain GABA and alanine levels in acute studies, the PEH analogues (at 30 mg/kg i.p.) exhibited differing neuroprotective efficacies, with FPEH being the only one of the analogues to exhibit neuroprotective activity *in vivo* when administered three hours post-stroke and then once daily for 7 days in gerbils. FPEH was also more effective than MePEH and MeOPEH at reducing glutamate levels, which may account for its neuroprotective effect. The PEH analogues showed little or no activity in the DSP-4-induced hippocampal depletion model in the rat.

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LIST OF ABBREVIATIONS

α-KG	α-ketoglutarate
5HT	5-hydroxytryptamine (serotonin)
Ala	alanine
Ala-T	alanine transaminase
ANOVA	analysis of variance
Ci	Curie
CNS	central nervous system
CSF	cerebrospinal fluid
DEP	deprenyl
DSP-4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine HCl
FPEH	4-fluorophenylethylidenehydrazine
g	gram
GABA	γ-aminobutyric acid
GFAP	glial acidic fibrillary protein
HCl	hydrochloric acid/hydrochloride
hr	hour
HP	Hewlett Packard
HPLC	high pressure liquid chromatography
i.p.	intraperitoneal(ly)
kg	kilogram
L	litre
m	metre

M	molar
MAO	monoamine oxidase
mg	milligram
min	minute
mL	millilitre
mm	millimetre
MePEH	4-methylphenylethylidenehydrazine
MeOPEH	4-methoxyphenylethylidenehydrazine
NA	noradrenaline
ng	nanogram
nM	nanomolar
PEA	β-phenylethylamine
PEH	phenylethylidenehydrazine
PLZ	phenelzine
s	second
TRIS	Tris buffer
veh	vehicle
μL	microlitre
μM	micromolar
°C	degrees Celsius
VPA	valproic acid
VGB	vigabatrin

Chapter 1

General Introduction

1.1 Phenelzine

Phenelzine (β-phenylethylhydrazine; PLZ) (Fig. 1) is a hydrazine-containing compound which, in addition to being a nonselective inhibition of monoamine oxidase (MAO), also inhibits GABA-transaminase (GABA-T) and alanine transaminase (ala-T) (Popov and Matthies, 1969; Tanay et al., 2001b; Kennedy et al., 2005). It is used in the treatment of depression with or without comorbid anxiety and has also been reported to be effective in panic disorder and social anxiety disorder (Ballenger, 1986). Intraperitoneal administration of PLZ to rats results in dose- and time-related changes in brain levels of several biogenic amines as well as the amino acids GABA and alanine (Popov and Matthies, 1969; Philips and Boulton, 1979; Baker et al., 1991; Wong et al., 1991; Tanay et al., 2001; Parent et al., 2002). It has been demonstrated to cause changes in abundance of mRNA for some GABA receptor subunit isoforms after chronic administration (Tanay et al., 2001a). Its anxiolytic properties appear to be due, at least in part, to its ability to increase brain levels of GABA (Paslawski et al., 2001). It is also of interest that, like several other GABAergic agents (Yang et al., 2000; Chen et al., 2000; Iqbal et al., 2002), PLZ is protective against neuronal damage caused by global ischemia in the gerbil (Todd et al., 1999; Tanay et al., 2002).

With respect to its interaction with MAO, PLZ is interesting in that not only is it an inhibitor of MAO, but it is also a substrate for that enzyme (Clineschmidt & Horita, 1969a, 1969b; Baker et al., 2000; Holt et al., 2004). Possible metabolites of PLZ formed via the action of MAO on PLZ include phenylacetic acid and

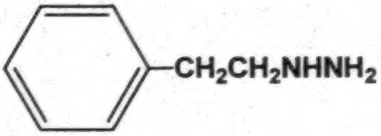

CH2CH2NHNH2

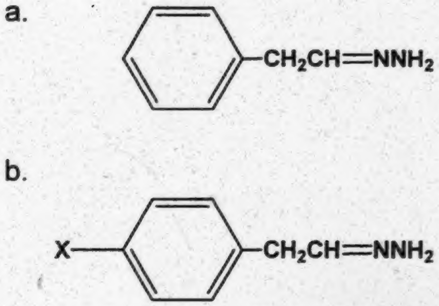
Figure 1. Chemical structure of phenelzine (PLZ).

phenylethylidenehydrazine (Tipton, 1972; Clineschmidt and Horita, 1969a,b; Baker et al., 1999).

While the physiological relevance and effects of these metabolites are largely unknown, it has been shown that inhibition of MAO-mediated metabolism of PLZ prevents the PLZ-associated increase in brain GABA levels in rats (Popov and Matthies, 1969; Todd and Baker, 1995). Tranylcypromine (a nonselective MAO inhibitor) and *l*-deprenyl (a selective MAO-B inhibitor) prevented the PLZ-associated increase in brain GABA levels when administered to rats prior to the administration of PLZ (Popov and Matthies, 1969; Todd and Baker, 1995). These findings suggest that one or more metabolites of PLZ play an important role in the GABA-elevating effect of that drug. At the present time the metabolite(s) responsible for the effect have not been identified, but phenylethylidenehydrazine (PEH; Fig. 2) is a likely candidate. Phenylacetic acid and *p*-hydroxyphenylacetic acid, other potential metabolites (Popov and Matthies, 1969), do not inhibit GABA-T or elevate brain levels of GABA (Baker, personal communication), but PEH has been synthesized in our laboratories and has been shown to dose- and time-dependently inhibit GABA-T activity and increase brain GABA levels in rats following intraperitoneal injection (Paslawski et al., 2001). Unlike the parent drug, PEH causes only a weak and short-lived inhibition of MAO. However, in common with PLZ, PEH has also been demonstrated to have neuroprotective effects in the global ischemia model in the gerbil, although it is somewhat weaker (Tanay et al., 2002). PEH has a potential clinical advantage over PLZ as a neuroprotective agent since, because of its weak effect on MAO-A, it should not produce the hypertensive effect that is sometimes seen with PLZ when it is taken by patients who subsequently ingest foods containing sympathomimetic amines such as tyramine (Murphy et al., 1984). It is also a much stronger inhibitor of GABA-T than is vigabatrin (γ-vinyl-GABA) (Todd and Baker, submitted) and thus, like vigabatrin, could be an effective anticonvulsant. Vigabatrin is used clinically, but high doses are required and it has the disadvantage of causing some ophthalmologic side effects (Sills, 2003; Duboc et al., 2004).

Given the previously reported neuroprotective actions of PLZ and PEH and the possibility of PEH being metabolised at the *para* position of its phenyl ring (McKenna et al., 1990), novel analogues of PEH, namely 4-fluoro-PEH, 4-methyl-PEH and 4-methoxy-PEH (Fig. 2) were synthesized to determine if they retained the neuroprotective properties of PEH and PLZ. Since these are new compounds, some of their pharmacological/neurochemical properties, particularly effects on brain levels of GABA (and other amino acids), brain activity of GABA-transaminase and alanine transaminase, body temperature and on cytochrome P450 (CYP)-mediated metabolism were studied.

Part of the remainder of the introduction will deal with GABA and stroke because of their relevance to the research projects described here. For the sake of completeness, GABA receptors and transporters will be described briefly, although only GABA metabolism and brain levels were investigated in the current project.



Substitution at X	Compound
F-	FPEH
CH_3-	MePEH
CH_3O-	MeOPEH

Figure 2. Chemical structures of phenylethylidenehydrazine (a. PEH) and the PEH analogues (b) 4-fluorophenylethylidenehydrazine (FPEH), 4-methylphenylethylidenehydrazine (MePEH) and 4-methoxyphenylethylidenehydrazine (MeOPEH).

1.2 GABA Receptors

Gamma-aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the mammalian CNS. Its physiological effects are mediated by plasma membrane receptor proteins, of which there are three types: $GABA_A$, $GABA_B$ and $GABA_C$.

Structurally, $GABA_A$ receptors are pentamers of various combinations of α, β, ρ, γ, δ, ε and π subunits. To date, 6 α, 3 β, 3 ρ, 3 γ and one each of δ,ε, θ and π subunits have been identified in mammals (Wafford, 2005). The most common subunit combination in rodents is 2 α subunits, 2 β subunits and 1 γ subunit. All of the subunits possess a long, extracellular N-terminus, 4 transmembrane (TM) domains, a large TM3-TM4 intracellular loop and a short extracellular C-terminal domain (Nestler et al., 2001). In addition to the GABA binding site, the $GABA_A$ receptor complex also has binding sites for several other compounds including steroids, benzodiazepines, barbiturates, alcohol, picrotoxin, and zinc (Dunn et al., 1994; DeLorey and Olson, 1999; Cooper et al., 2003; Nestler et al., 2001). Agonists at $GABA_A$ receptors include GABA, muscimol, and 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP). Bicuculline is a competitive antagonist at the $GABA_A$ receptor, while picrotoxin is a non-competitive antagonist, blocking permeation of the intrinsic ion channel by chloride ions (Cooper et al., 2003; Czuczwar and Patsalos, 2001). $GABA_A$ and $GABA_C$ receptors are ligand-gated ion channel-associated receptors which allow the passage of chloride ions across the cell membrane following the receptor activation. While $GABA_A$ receptor channel-associated chloride flux is linked to activation of the receptor by GABA and muscimol and inhibited by bicuculline, the $GABA_C$ receptor is resistant to bicuculline. Whereas $GABA_A$ receptors are ubiquitously distributed throughout human and rodent brains, $GABA_C$ receptors are largely concentrated in the retina, but are also present in cerebellum, hippocampus, optic tectum and spinal cord (Czuczwar and Patsalos, 2001; Nestler et al., 2001; Frolund et al., 2002; Cooper et al., 2003). $GABA_A$ receptors have been implicated in anxiety, seizure activity, schizophrenia, depression, state of consciousness and body temperature (Jha et al., 2001; Schwartz-Bloom and Sah, 2001; Varju et al., 2001; Brambilla et al., 2003; Cupello, 2003; Wassef et al., 2003; Costa et al., 2004; Coyle, 2004; Galeffi et al., 2004).

$GABA_B$ receptors are metabotropic, guanine nucleotide binding protein (G-protein)-coupled receptors, can be found both pre- and post-synaptically and are linked to G-protein-mediated modulation of the transmembrane flow of potassium and calcium ions (Nestler et al., 2001; Cooper et al., 2003). Activation of pre-synaptic $GABA_B$ receptors results in inhibition of calcium ion influx while activation of post-synaptic $GABA_B$ receptors is associated with activation of potassium channel-mediated potassium efflux. The result of $GABA_B$ receptor activation is a reduction in neurotransmitter release (presynaptic receptors) or a reduction in neuronal excitability (post-synaptic receptors) (Cooper et al., 1993). $GABA_B$ receptors have also been described on glial cells (Charles et al., 2003; Kuhn et al., 2004), where their roles are only now being determined. Activation of microglial $GABA_B$ receptors results in inhibition of the release of the pro-inflammatory cytokines interleukin-6 (IL-6) and -12 (IL-12) (Kuhn et al., 2004).

The exact mechanisms by which these effects of $GABA_B$ receptor activation occur have not yet been elucidated. GABA and baclofen are agonists at $GABA_B$ receptors, while phaclofen is an antagonist at these receptors (Cooper et al., 2003).

1.3 GABA Transporters

GABA reuptake is mediated by transporter proteins located in the plasma membranes of neurons and glia. GABA transporters (GATs), of which there are three main types (GAT-1 to GAT-3), are part of a family of sodium and chloride-dependent transporters and differ with respect to their ligand specificity and cellular distributions (Schousboe, 2000; Gadea and Lopez-Colome, 2001; Dalby, 2003). Tiagabine and β-alanine are inhibitors of GABA reuptake processes, with tiagabine being more specific in this regard (Dalby, 2003; Schousboe, 2003).

1.4 GABA Metabolism

A number of different enzymes and cell types are involved in the metabolism of GABA. The pathway in which these enzymes take part is referred to as the GABA shunt (Fig. 3) and allows α-ketoglutarate (α-KG) to bypass the Krebs/tricarboxylic acid (TCA) cycle and, instead, be converted to glutamate by the enzyme GABA-transaminase (GABA-T). The other enzymes involved in the GABA shunt pathway are glutamic acid decarboxylase (GAD), succinic semialdehyde dehydrogenase, glutamine synthetase, and glutaminase (Cooper et al., 2003).

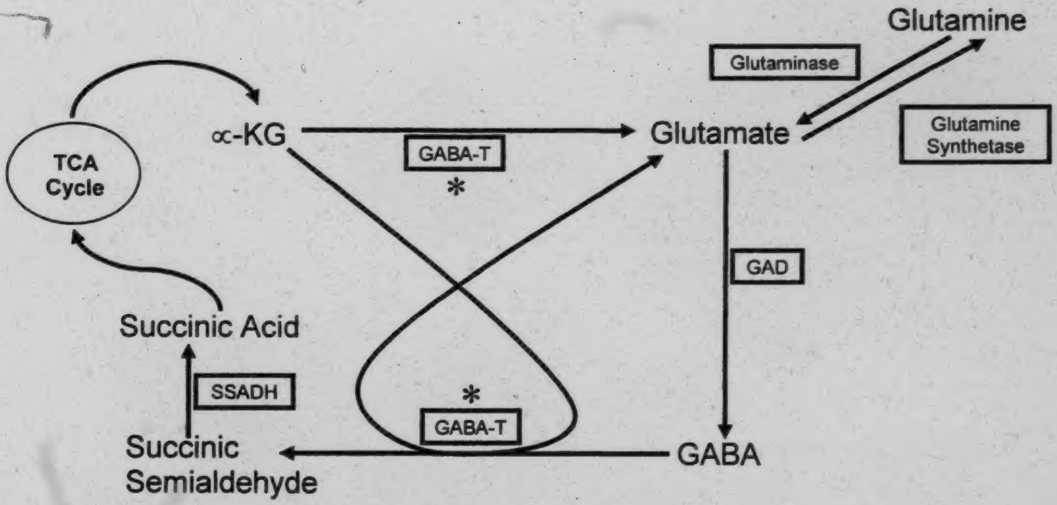

Glutamine
Glutaminase
Glutamine Synthetase
TCA Cycle
∝-KG
GABA-T
*
Glutamate
GAD
Succinic Acid
SSADH
*
GABA-T
Succinic Semialdehyde
GABA

Figure 3. The GABA Shunt. Schematic of the GABA shunt, illustrating the conversion of GABA to succinic semialdehyde (SSA) by the enzyme GABA-transaminase. α-KG = α-ketoglutarate.

GABA-T catalyzes an oxidative transamination reaction in which α-ketoglutarate acts as an amine acceptor and is transformed to glutamate, while GABA acts as an amine donor as it is converted to succinic semialdehyde. Pyridoxal 5'-phosphate (PLP), the biologically active form of vitamin B_6 (pyridoxine; Tunnicliff and Ngo, 1998) is a cofactor for this reaction. GABA-T, a mitochondrial membrane-associated enzyme, is ubiquitously distributed in the body (Sherif and Ahmed, 1995; Cooper et al., 2003; Sarup et al., 2003). GAD, a cytoplasmic, PLP-requiring enzyme, catalyzes the conversion of glutamate to GABA, and has only been identified in GABA-synthesizing neurons (Soghomonian and Martin, 1998; Cooper et al., 2003). Two forms of GAD, GAD_{65} and GAD_{67}, have been identified and differ with respect to molecular weights and their affinities for PLP. Although GAD_{65} has a higher affinity for PLP than does GAD_{67}, both GAD enzymes display lower affinity towards the cofactor than does GABA-T (Cooper et al., 2003).

Glutamine synthetase (GS) is found in astrocytes, glial cells involved in several aspects of brain function including maintenance of the extracellular environment, support for the metabolic needs of neurons and maintenance of the blood-brain barrier. Glutamine synthetase catalyzes the conversion of glutamate to glutamine following uptake of the former by astrocytes. This newly-formed glutamine serves as a sink for excess ammonia and as a storage and transport form of glutamate. Glutamine can be released by astrocytes and taken up by neurons, where it is then converted back to glutamate via the action of glutaminase. The liberated glutamate can then be released, shuttled into the tricarboxylic acid cycle (also referred to as the TCA cycle, Krebs Cycle or Citric Acid Cycle) or re-packaged into exocytotic vesicles for later depolarization-associated neurotransmitter release (Westergaard et al., 1995).

The enzymes of the GABA shunt (Fig. 3) are potential targets for manipulation of brain levels of GABA and glutamate. However, because of the interconnections between the metabolic pathways for many amino acids, the results of changing the activities of such enzymes can often be quite complex. For example PLP, in addition to acting as a cofactor for GAD, is also an obligatory cofactor for GABA-T, alanine-transaminase (ala-T), and other transaminase enzymes. Conditions affecting the availablility of metabolically active PLP have the potential to affect all PLP-dependent enzymes and, thus, brain levels of several amino acids (Tunnicliff and Ngo, 1998; Holt et al., 2004). Holt et al. (2004) suggest that humans possess as many as 48 PLP-dependent enzymes, almost half of which are transaminase/aminotransferase enzymes.

GABA-T can be inhibited by hydrazines and aminooxyacetic acid (Lightcap and Silverman, 1996; Tunnicliff and Ngo, 1998). Vigabatrin (a clinically used anticonvulsant) also inhibits GABA-T activity in an irreversible manner, causing inactivation of the enzyme (Czuczwar and Patsalos, 2001). In this regard, it is of interest that vigabatrin has been reported to be neuroprotective in the gerbil model of global ischemia (Shuaib et al., 1992, 1996), and that both PLZ and PEH are much more potent than vigabatrin at inhibiting GABA-T *ex vivo* (Todd and Baker, unpublished).

1.5 Ischemic Stroke

The term ischemic stroke is commonly used in reference to both the death of brain tissue as a result of inadequate oxygen supply, and the clinical manifestations of this occurrence (Allen and Lueck, 1999). The reduction in oxygen supply may be due to a reduction in or cessation of blood flow to a particular region of the brain (i.e. focal cerebral ischemia) or it may result from a generalized cessation of blood flow as occurs in cardiac arrest (global ischemia; Green et al., 2003). Ischemic stroke can be subdivided into several categories based on the mechanism by which the ischemic insult occurs.

Ischemic stroke may arise due to: (1) clot (thrombus) formation within a cerebral vessel, resulting in compromised blood flow through the vessel; (2) occlusion of a cerebral vessel by a thrombus that formed elsewhere in the body and then traveled (as an embolus) to a cerebral vessel, becoming lodged in the vessel and thereby compromising blood flow; (3) occlusion of a cerebral blood vessel by a non-platelet plug that has gained entry into the circulation as a result of some traumatic event; or (4) external compression of a cerebral vessel by pathological structures such as tumours. Of these, the most common cause of ischemic stroke in humans is thrombus-associated interference with blood flow (Allen et al. 1999). The cerebral sites most commonly occluded by thrombi are the branches of the middle cerebral artery. These branches supply oxygen and nutrients to the superolateral temporal lobe (cortex), inferolateral frontal lobe (cortex) and striatum. Accordingly, symptoms of strokes in the territory supplied by the middle cerebral artery include speech and motor deficits (slurred speech, limb weakness or paralysis), but will vary depending on the specific vessel(s) occluded (Young and Young, 1997; Allen et al., 1999).

A significant number of stroke victims will survive long-term, but be faced with long-term disability, making them unable to live completely independent of others with respect to daily tasks of living. Because of the sequelae of major vessel occlusion in the brain, stroke continues to be a leading cause of disability in North America and worldwide (Lo et al., 2003). Individuals surviving the initial insult and resultant neuronal loss often face complications such as pneumonia, venous thromboembolism, cardiac disease, seizures and depression (Muir, 2001). It is generally believed that improvement in the outcome following stroke will be achieved by a reduction in neuronal loss (i.e. neuronal preservation) and will be reflected by a better long-term survival following stroke, decreased sensorimotor deficits following stroke and more rapid recovery as a whole, following a stroke event. To this end, the focus of stroke research to date has largely been on improving neuronal survival following transient cerebral ischemia (Lo et al., 2003). This work relies heavily on a thorough understanding of the pathophysiological events that occur in the brain and in the body as a whole following a cerebral ischemic insult.

At one time glutamate was believed to be the primary target at which stroke interventions should be aimed. Accumulating evidence now suggests that three sets of processes are particularly important in stroke pathophysiology, namely excitotoxicity, inflammation and apoptosis (Schwartz-Bloom and Sah, 2001; Dirnagl et al., 2003). Specific processes such as GABAergic system activity, glutamate receptor activation, interleukin release and caspase activation are all part of the general picture, and can each be fitted into one or more of those three categories. For example, GABAergic activity is believed to play a role in preventing or decreasing excitotoxic damage (Green et al., 2000; Schwartz-Bloom and Sah, 2001), but recent evidence also suggests a role for GABA in modulating inflammatory responses (Tian et al., 1999; Kuhn et al., 2004).

1.6 Glutamate in Ischemic Stroke

1.6.1 Glutamate Receptors

Glutamate is a major excitatory transmitter in mammals, exerting its physiologic effects via ionotropic, ion channel-coupled receptors and metabotropic, G-protein-coupled receptors (Rivera-Cervantes et al. 2004).

There are 3 classes of ionotropic glutamate receptors, the α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors, kainate receptors, and N-methyl-D-aspartate (NMDA) receptors, named after their selective agonists, AMPA, kainic acid and NMDA, respectively (Bigge, 1999; Rivera-Cervantes et al., 2004). All ionotropic receptor subunits possess a long extracellular N-terminus, 3 transmembrane domains, a membrane-loop domain and a variable length, intracellular C-terminus (Bigge, 1999; McFeeters and Oswald, 2004). AMPA receptors generally mediate the fast component of glutamate-induced post-synaptic depolarization, while NMDA receptor currents are more latent, occurring after the AMPA-receptor-associated depolarization, which relieves NMDA receptors of their membrane voltage-dependent magensium blockade (Bigge, 1999). AMPA, kainate and NMDA receptors are permeable to Na^+; however, NMDA receptors are also permeable to Ca^{2+}, and Ca^{2+} permeability is also seen with AMPA receptors lacking the GluR2 subunit (Arundine and Tymianski, 2003).

Metabotropic glutamate receptors (mGluRs) are part of the family of G-protein-coupled receptors and are thus linked to intracellular signalling pathways via heterotrimeric G-protein complexes (Maiese et al., 1999). To date, 8 mGluR subtypes have been identified and cloned (Dingledine and McBain, 1999). The mGluRs can be subdivided into three groups, which differ with respect to their physical locations on cell membranes (i.e. presynaptic vs. post-synaptic vs. extra-synaptic; Takumi et al., 1999) and effects on intracellular Ca^{2+} concentrations (Maiese et al., 1999).

1.6.2 Glutamate in Ischemia

Ischemia results in depletion of adenosine triphosphate (ATP) stores as well as reduction and eventual cessation of ATP production (Juurlink and Sweeney, 1997; Lipton, 1999). As a consequence, ATP-dependent processes are compromised, resulting in a general loss of cellular homeostasis. These ATP-dependent processes include plasma membrane transport processes such as the Na^+/K^+-ATPase, an ATP-driven pump that maintains the inward- and outward-directed sodium and potassium gradients, respectively. Loss of this activity leads to the passive movement of sodium ions into neurons and eventual depolarization of those cells and the release of glutamate (Juurlink and Sweeney, 1997; Lipton, 1999; Lo et al., 2003). Liberated glutamate activates AMPA receptors, which results in continued depolarization as well as the removal of NMDA receptor voltage-dependent magnesium block, thereby activating NMDA receptors. Excessive entry of calcium ions into neurons through NMDA receptor-associated channels as well as through GluR2 subunit-deficient AMPA receptor-associated channels (Arundine and Tymianski, 2003; Rivera-Cervantes et al., 2004) are thought to be among the principal triggers of the damage seen in ischemia. The contribution of mGluRs to changes in intracellular calcium concentrations in the setting of excessive, unchecked glutamatergic activity must also be considered, as mGluR activation affects Ca^{2+} release from intracellular stores (Maiese et al., 1999) as well as Ca^{2+} entry through plasma membrane Ca^{2+} channels (Foreman et al., 2005; Guo et and Ikeda, 2005, Lu and Rubal, 2005). Excessive calcium buildup intracellularly leads to pathological activation of calcium-dependent enzymes, including nitric oxide synthase, proteases, lipases and endonucleases (Arundine et al., 2003) as well as to further depolarization and glutamate release. Oxygen deficit uncouples the reactions of glycolysis from oxidative phosphorylation, resulting in excessive generation of free radicals, as does unchecked activity of calcium-dependent enzymes such as phospholipase A_2 and other enzymes such as MAO. Free radical damage to lipids in intracellular and plasma membranes contributes to the disruption of cellular homeostasis and also leads to altered function of plasma membrane and cytoplasmic transport proteins and enzymes. The entire cascade of events resulting from excessive glutamate receptor activation and consequent calcium overload is referred to as glutamate excitotoxicity. The dysregulation of cellular processes and loss of glutamate in rodent brains increases in response to ischemia (Juurlink and Sweeney, 1997; Green et al., 2000, Schwartz-Bloom and Sah, 2001; Leker and Neufeld, 2003).

1.7 GABA in Ischemic Stroke

Because of the ability of GABA, acting via GABA_A receptors, to hyperpolarize cell membranes of mature neurons, it has been suggested that drugs that increase GABA levels or that act as GABA_A receptor agonists may be useful in clinical conditions where deficits of GABAergic activity or excessive glutamatergic activity play important roles in the pathophysiology (Shuaib and Kanthan, 1997; Shuaib et al., 1997). Two such conditions include epilepsy (Czuczwar and Patsalos, 2001; Frolund et al., 2002) and cerebral ischemia.

One cardinal neurochemical finding in ischemic brain tissue is a pathological increase in extracellular glutamate levels in areas experiencing compromised blood flow and consequent failure of oxidative phosphorylation (Kanthan et al., 1995; Juurlink and Sweeney, 1997; Lipton, 1999; Yang et al., 2001; Iqbal et al., 2002; Lo et al., 2003). Failure of oxidative phosphorylation (production of ATP) results in a time-dependent loss of homeostasis due to failure of ATP-dependent processes, including calcium extrusion from the intracellular space by calcium pumps. This homeostatic failure leads, eventually, to depolarization and uncontrolled neuronal release of neurotransmitters such as glutamate. Excessive glutamatergic activity results in unchecked depolarization, generation of free radicals by calcium-dependent enzymes and disorganized metabolic pathways (i.e. uncoupled oxidative metabolism), free radical-mediated damage and eventual cell death (Juurlink and Sweeney, 1997; Lipton, 1999).

$GABA_A$ receptor activation and consequent hyperpolarization physiologically antagonizes glutamate receptor activation-associated depolarization in mature neurons. Several GABAergic agents have been examined in the laboratory setting for their abilities to attenuate glutamate-mediated excitotoxicity. These compounds include GABA receptor modulators such as diazepam (Schwartz et al., 1995; Hall et al., 1998; Schwartz-Bloom et al., 2000), GABA agonists such as muscimol (Lyden et al., 2000), GABA-T inhibitors such as vigabatrin (Costa et al., 2004), PLZ and PEH (Todd et al., 1999; Tanay et al., 2002), GABA reuptake inhibitors such as tiagabine (Iqbal et al., 2002) and other potentiators of GABA activity such as clomethiazole (Shuaib et al., 1995; Sydserff et al., 2000). All of the compounds listed above have demonstrated efficacy *in vitro* and/or *in vivo* in reducing ischemia-associated neuronal cell death (Chen et al., 2000; Iqbal et al., 2002; Schwartz-Bloom and Sah, 2001).

1.8 Hypothermia in Ischemic Stroke

Clinical studies have shown that stroke patients with elevated core temperature also have a poorer prognosis for recovery relative to normothermic patients (Zaremba, 2003; Hanchaiphiboolkul, 2005) and preclinical studies of cerebral ischemia in rats have demonstrated increased infarct size in hyperthermic animals relative to normothermic animals (Noor et al., 2003).

Hypothermia in stroke situations reduces swelling (Kurokawa et al., 2001), intracranial pressure (Li et al., 1999; Feigin et al., 2002; Bernard and Buist, 2003), the release of excitatory amino acids (Feigin et al., 2002; Bernard and Buist, 2003) and metabolic rate and energy requirements (Bernard and Buist, 2003; Erecinska et al., 2003). Hypothermia has many effects on cerebral metabolism, including a reduction in ATP metabolism and reductions in the rates of the glycolytic and tricarboxylic acid pathways, thereby reducing the utilization of glucose and the formation of lactate (Bernard and Buist, 2003); both of these effects would be beneficial in the setting of cerebral ischemia.

The number of GABAergic agents demonstrated to induce hypothermia is growing and now includes diazepam (Davies et al., 2004), tiagabine (Iqbal et al., 2002; Inglefield et al., 1995) muscimol, THIP, nipecotic acid, and GABA itself (Frosini et al., 2004). In the setting of experimental cerebral ischemia, the hypothermia-inducing effects of tiagabine and diazepam were also shown to contribute to their neuroprotective activities (Iqbal et al., 2002; Kuhmonen et al., 2002, Davies et al., 2004). Although the molecular mechanisms by which the hypothermic effects are elicited have not been identified, on a more gross level the effects have been linked to the medial preoptico-anterior hypothalamus (mPOAH)(Jha et al., 2001) and the raphé pallidus (Zaretsky et al., 2003).

In light of the above, it is important for the hypothermia-inducing effects of GABAergic agents to be examined. PLZ was also included in these experiments.

1.9 Animal Models of Ischemia and Neuronal Toxicity

In studying the neuroprotective potential of pharmacologic agents, animal models are of tremendous importance. While *in vitro* studies allow dissection of pathophysiological events and identification of pharmacologic targets, *in vivo* testing allows for examination of the true potential of compounds in intact organisms, where organ system interactions and hormonal, blood pressure, temperature, and idiopathic changes come into play. In evaluating the effects of potential neuroprotective agents, several models have been employed. The two used in the projects described in this thesis are the gerbil model of global ischemia (transient forebrain ischemia) and the *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzyl amine (DSP-4) model of noradrenergic neuronal toxicity in the rat hippocampus.

The models believed to most accurately reproduce the pathology seen in focal human stroke are those in which specific rat cerebral vessels are occluded, resulting in focal lesions (Small and Buchan, 2000; Green et al., 2003). In these models blood flow through vessels is generally interrupted by introduction of a fibrin clot into the vessel (via use of a cannula) or by transient, mechanical occlusion using a silk suture or external compression of the vessel(s). While the rat focal ischemia models (Ginsberg and Busto, 1989; Small and Buchan, 2000) are the most reflective of thromboembolic stroke in humans, they are not as well-suited for use as initial rapid screens for neuroprotective activity as is the gerbil transient ischemia model. In this regard, the gerbil model of transient forebrain ischemia is often used for the initial identification of potential neuroprotective agents. In addition, Todd and colleagues found no neuroprotective effect with PEH in the rat focal ischemia model (Todd, Shuaib and Strel, unpublished).

Global ischemia in humans occurs in settings including myocardial infarction, carbon monoxide poisoning, drowning, birth asphyxia and respiratory arrest (e.g. due to drug overdose). The pathology seen in these types of insults has been reproduced in rodent models of birth asphyxia (hypoxia-ischemia) and in rodent models of global cerebral ischemia including the gerbil model of transient forebrain ischemia (unilateral and bilateral occlusion of the CCAs) and rat models of global cerebral ischemia (four vessel occlusion or 2 vessel occlusion with hypotension) (Pulsinelli and Brierly, 1979; Smith et al., 1984; Ashwal and Pearce, 2001). Many of these models incompletely reflect the etiology of the human situations listed above in that the rodent models render only the brains of the animals hypoxic, while in human models the entire body is made hypoxic. Accordingly, in the rodent models dysfunction of the liver, kidneys, heart and other peripheral organs are not induced.

The cerebral circulation of the Mongolian gerbil differs from rats and humans in that in gerbils the posterior communicating arteries (part of the Circle of Willis) and compensatory routes for blood flow are absent or malformed (Levine and Payan, 1966; Levine and Sohn, 1969; Kahn, 1972; Berry et al., 1975; Ginsberg and Busto, 1989; Small and Buchan, 2000). As a result, bilateral occlusion of the common carotid arteries (CCAs) of the gerbils results in complete ischemia of the entire forebrain. Nonetheless, these models are convenient for the examination of pathophysiology and potential for pharmacological intervention following a global cerebral insult. To this end, one of the most commonly-employed models for the examination of neuroprotective effects following cerebral ischemia is the gerbil model of transient forebrain ischemia.

In gerbils, bilateral occlusion of the CCAs (by external pressure using surgical clips and/or sutures) for five minutes, followed by reperfusion (re-establishment of blood flow by removal of external pressure from the carotid arteries) results in a reproducible pattern of ischemic damage (Kirino, 1982; Ginsberg and Busto, 1989; Small and Buchan, 2000). The CA1 region of the hippocampus is the most sensitive to this insult, with cell death occurring over the course of 4 days to one week (Kirino, 1982). The initial cellular effects of the ischemic insult (cytoplasmic vacuolation, darkening of soma; referred to as ischemic cell changes) are visible in the hippocampi of these animals within hours (Kirino, 1982). In gerbils subjected to transient forebrain ischemia, hyperactivity was typically observed for 1-2 days following the operation.

There has been some debate as to the mechanisms responsible for the delayed neuronal death seen following bilateral occlusion of the CCAs, with some studies reporting evidence of apoptosis that can be prevented using caspase inhibitors (Gillardon et al., 1999; Ouyang et al., 1999; Jover et al., 2002; Abe et al., 2004), while others have refuted those claims, suggesting instead that the patterns of cell death more accurately reflect necrosis (Colbourne et al., 1999). Despite the controversy regarding the predominant cell death pathway activated by transient global ischemia, several observations have been made regarding changes seen following global ischemia, and those observations implicate increased expression of calcium-permeable glutamate receptors (Bennett et al., 1996; Aronica et al,, 1998), intracellular accumulation of calcium ions (Nakamura et al., 1999) and immune/inflammatory responses (Lin et al., 1998; Stoll et al., 1998; Schafer et al., 2000; Kim et al., 2003; Sugawara et al., 2002; Danton and Dietrich, 2003) in the cellular damage seen following transient forebrain ischemia. Recently, roles for cyclooxygenase enzymes (Candelario-Jalil et al., 2003) and intracellular accumulation of zinc has also been suggested (Caldarone et al., 2004). Observations such as these warrant the continued investigation of compounds for anti-inflammatory and anti-excitotoxic effects. At the present time clomethiazole (Sydserff et al., 2000; Chaulk et al., 2003), benzodiazepines (Dowden et al., 1999; Schwartz-Bloom et al., 2000), tiagabine (Iqbal et al., 2002), topiramate (Lee et al., 2000), l-deprenyl (Lahtinen et al., 1997), tetracyclines (Yrjanheikki J et al., 1998), calcium channel antagonists (Zapater et al., 1997), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor antagonists (Kawasaki-Yatsugi et al., 1997), nitric oxide synthase inhibitors (Kohno et al., 1997), lamotrigine (Shuaib et al., 1995) and hypothermia (Buchan and Pulsinelli, 1990; Colbourne and Corbett, 1994; Corbett et al., 1997; Colbourne et al., 1998), among others, have been shown to reduce neuronal death following transient forebrain ischemia in rodents.

The toxin DSP-4 damages neurons by forming covalent bonds with sulfhydryl groups and its selectivity for noradrenergic neurons is a result of its uptake into those neurons via noradrenaline (NA) transporters (Magyar and

Szende, 2004). While inhibition of NA reuptake represents one potential mechanism by which neurons may be protected from the toxic effect of DSP-4 (Ross, 1976; Jonsson et al., 1982; Berry et al., 1994), other studies suggest that inhibition of MAO-B may also be important in preventing DSP-4-associated neurotoxicity, although the mechanism is not yet clear (Gibson, 1987; Yu et al., 1994). Because of the heterogeneity of the pharmacological effects of several of the drugs that have shown neuroprotective activity in the DSP-4 model and the absence of definite identification of the essential mechanisms required for a compound to be neuroprotective in this model, it is reasonable for novel compounds with potential neuroprotective activity to be screened for neuroprotective activity in this assay and, indeed, this model has become a popular routine screen for neuroprotective agents (Yu et al., 1994). Accordingly, the PEH analogues under investigation here were examined for their effectiveness in this model.

1.10 Cytochrome P450 Enzymes

Cytochrome P450 monooxygenase is one of the major enzyme systems in the body and is responsible for the metabolism of a large number of endogenous and exogenous chemicals (Parkinson, 1996). A major component of the enzyme is a hemoprotein called cytochrome P450 (CYP) (Hlavica et al., 2003).

The name cytochrome P450 stems from the fact that the cytochrome, which was initially identified as a red liver pigment (P), displays a spectral absorbance maximum at approximately 450 nm when reduced and complexed with carbon monoxide. It is now clear that the CYPs represent a family of enzymes rather than a single enzyme (Gonzalez, 1989; Nelson et al., 1993). The prefix "CYP" is used to designate the cytochrome P450 system (Nebert et al., 1987), and CYP enzymes with less than 40% amino acid sequence identity are assigned to different gene families. Gene families are designated by Arabic numerals after the CYP, and subfamilies are designated by a capital letter [members of the same subfamily have greater than 55% amino acid sequence identity and appear to be within the same cluster on a chromosome (Schenkman, 2000)]. Individual enzymes within a subfamily are designated by terminal Arabic numbers corresponding to the chronological order of their discovery (Nelson et al., 1996; Parkinson, 1996). As an example, in CYP2D6, CYP represents cytochrome P450, 2 the gene family, D the subfamily, and 6 the individual enzyme.

Fourteen CYP gene families have been identified to date. These 14 families comprise 26 subfamilies, of which 20 have been mapped in the human genome (Nelson et al., 1996). The 14 gene families are 1, 2, 3, 4, 5, 7, 8, 11, 17, 19, 21, 24, 27 and 51. Families 1, 2 and 3 are those primarily involved in drug biotransformation (Gibson and Skett, 2001).

Of the CYP enzymes, the ones that play prominent roles in drug metabolism include CYPs 3A4, 2D6, 2C9, 2C19 and 2B2 (Meyer, 1996; Parkinson, 1996). CYP3A4 is one of the most prominent CYP enzymes in the body, and substrates include several benzodiazepines as well as nifedipine, erythromycin, venlafaxine and zolpidem. CYP2D6 accounts for only 1-5% of total CYP hepatic protein mass, but is responsible for metabolism of numerous drugs, including amphetamine, dextromethorphan, several phenothiazine antipsychotics, tricyclic antidepressants, deprenyl, fluoxetine and olanzapine. CYP2C19 accounts for approximately 1% of hepatic protein mass, and important substrates include several barbiturates, S-mephenytoin, diazepam and the antidepressants imipramine, citalopram and moclobemide. CYP1A2 catalyzes the metabolism of drugs such as acetaminophen, caffeine, clozapine, imipramine, propranolol and warfarin. Although CYP2B6 has a lower level of expression than the other CYP enzymes discussed in this thesis, it has been reported to be involved in the N-dealkylation of a number of important drugs such as S-mephenytoin, lidocaine, diazepam and temazepam and in the hydroxylation of the antidepressant bupropion (Pritchard and Wolf, 2000; Rush et al., 2005).

CYP enzymes catalyze the insertion of an active oxygen species into a substrate molecule using iron protoporphyrin IX, to which a heme group is non-covalently bound, as a prosthetic group. During the catalytic process, the hemoprotein is reduced from its ferric (Fe^{3+}) form to its ferrous (Fe^{2+}) form; the reducing equivalent required for this process is derived from NADPH (Gibson and Skett, 2001). Rearrangement of the intermediate which results in the formation of the final metabolite may involve retention or expulsion of the oxygen. Ring hydroxylation and N- and O-dealkylation are examples of common metabolic routes that are mediated by this system.

Numerous drugs act as substrates and/or inhibitors or inducers of CYP enzymes, and because of this there may be a high risk for pharmacologic drug-drug interactions when patients are taking multiple drugs, as is often the case in psychiatric or neurologic patients. Because of this potential problem, the Food and Drug Administration of the USA required in 1997 that henceforth information about interactions of new drugs with CYP enzymes would be a required part of drug development programs. In this thesis, I report a screen of PEH and its analogues for ability to inhibit CYP enzymes.

1.11 Thesis Objectives

The objectives of this thesis were to characterize novel PEH analogues with respect to their effects: a) on GABA-T, ala-T and brain amino acid levels; b) in two screens for neuroprotective agents, i.e. the gerbil global ischemia model and DSP-4-induced noradrenaline depletion in the rat; c) on CYP enzymes important in drug metabolism; and d) on body temperature in gerbils.

1.12 References

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Chapter 2

General Materials and Methods

2.1. GENERAL METHODS

2.1.1 Chemicals Used

Table 1. Chemicals used in the experiments described henceforth.

Chemical Name	**Supplier**
acetic acid (glacial)	Fisher Chemical (Fair Lawn, NJ)
acetonitrile, HPLC grade	Fisher Chemical (Fair Lawn, NJ)
^{3}H-L-alanine	Perkin Elmer (Boston Mass.)
γ-aminobutyric acid	Sigma Chemical Co. (St. Louis, MO)
^{3}H-γ-aminobutyric acid	Perkin Elmer (Boston Mass.)
2-aminoethylisothioluronium bromide	Sigma Chemical Co. (St. Louis, MO)
ammonium hydroxide	Fisher Chemical (Fair Lawn, NJ)
antibody: rabbit anti-GFAP	DAKO Corp. (Carpinteria, CA.)
antibody: goat anti-rabbit IgG	Sigma Chemical Co. (St. Louis, MO)
ascorbic acid	Fisher Chemical (Fair Lawn, NJ)
avidin biotin complex	DAKO Corp. (Carpinteria, CA.)
7-benzyloxy-4-(trifluoromethyl)-coumarin	Gentest (San Jose, CA)
chloroform	Fisher Chemical (Fair Lawn, NJ)
Contrad™	DeCon Labs (USA)
3-cyano-7-ethoxycoumarin	Gentest (San Jose, CA)
(-)-deprenyl HCl	Tocris (Ellisville, MO)
diaminobenzidine	Sigma Chemical Co. (St. Louis, MO)
dimethylsulfoxide	Fisher Chemical (Fair Lawn, NJ)
eosin yellow (aqueous)	Fisher Chemical (Fair Lawn, NJ)
7-ethoxy-4-(trifluoromethyl)-coumarin	Molecular Probes (Burlington, ON)
ethyl alcohol	Biochemistry Stores (Department of Biochemistry, U of A)
ethyl acetate	Fisher Chemical (Fair Lawn, NJ)
4-fluorophenylethylidenehydrazine	Dr. E.E. Knaus (Faculty of Pharmacy and Pharmaceutical Sciences, U of A)
glucose-6-phosphate dehydrogenase	Sigma Chemical Co. (St. Louis, MO)
α-D-glucose-6-phosphate, monosodium salt	Sigma Chemical Co. (St. Louis, MO)
Glutathione	Sigma Chemical Co. (St. Louis, MO)
Hematoxylin, Mayer's (Lillie's Modification)	DAKO Corp. (Carpinteria, CA.)
HEPES	Fisher Chemical (Fair Lawn, NJ)
hydrochloric acid	Fisher Chemical (Fair Lawn, NJ)
5-hydroxytryptamine	Sigma Chemical Co. (St. Louis, MO)
^{14}C-5-hydroxytryptamine	Perkin Elmer (Boston, Mass.)
Isopentane	Fisher Chemical (Fair Lawn, NJ)

α-ketoglutarate	Sigma Chemical Co. (St. Louis, MO)
L-alanine	Sigma Chemical Co. (St. Louis, MO)
L-DOPA	Sigma Chemical Co. (St. Louis, MO)
L-glycine	Sigma Chemical Co. (St. Louis, MO)
L-leucine	Sigma Chemical Co. (St. Louis, MO)
L-serine	Sigma Chemical Co. (St. Louis, MO)
L-glycine	Sigma Chemical Co. (St. Louis, MO)
L-asparagine	Sigma Chemical Co. (St. Louis, MO)
L-taurine	Sigma Chemical Co. (St. Louis, MO)
L-aspartic acid	Sigma Chemical Co. (St. Louis, MO)
L-glutamine	Sigma Chemical Co. (St. Louis, MO)
L-glutamate	Sigma Chemical Co. (St. Louis, MO)
magnesium chloride	Fisher Chemical (Fair Lawn, NJ)
4-methoxyphenylethylidenehydrazine	Dr. E.E. Knaus (Faculty of Pharmacy and Pharmaceutical Sciences, U of A)
7-methoxy-4-(trifluoromethyl)-coumarin	Gentest (San Jose, CA)
methyl alcohol	Fisher Chemical (Fair Lawn, NJ)
3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-hydroxy-4-methylcoumarin	Gentest (San Jose, CA)
4-methylphenylethylidenehydrazine	Dr. E.E. Knaus (Faculty of Pharmacy and Pharmaceutical Sciences, U of A)
nicotinamide adenine dinucleotide	Sigma Chemical Co. (St. Louis, MO)
nicotinamide adenine dinucleotide phosphate, monosodium salt	Sigma Chemical Co. (St. Louis, MO)
nicotinamide adenine dinucleotide, reduced	Sigma Chemical Co. (St. Louis, MO)
(-)-noradrenaline hydrochloride	Sigma Chemical Co. (St. Louis, MO)
perchloric acid	Fisher Chemical (Fair Lawn, NJ)
Permount^{TM}	Fisher Chemical (Fair Lawn, NJ)
phenelzine sulfate	Sigma Chemical Co. (St. Louis, MO)
phosphoric acid	Fisher Chemical (Fair Lawn, NJ)
phenylethylidenehydrazine	Dr. E.E. Knaus (Faculty of Pharmacy and Pharmaceutical Sciences, U of A)
o-phthaldialdehyde	Pierce (Rockford IL)
potassium chloride	Fisher Chemical (Fair Lawn, NJ)
potassium hydroxide	Fisher Chemical (Fair Lawn, NJ)
potassium phosphate	Fisher Chemical (Fair Lawn, NJ)
proteinase K	DAKO Corp. (Carpinteria, CA.)
pyridoxal phosphate	Sigma Chemical Co. (St. Louis, MO)
scintillation fluid (ReadySafe™)	Beckman Coulter (Fullerton, CA.)
sodium bicarbonate	Fisher Chemical (Fair Lawn, NJ)
sodium chloride	Fisher Chemical (Fair Lawn, NJ)
sodium hydroxide	Fisher Chemical (Fair Lawn, NJ)

sodium ethylinediaminetetraacetic acid	Fisher Chemical (Fair Lawn, NJ)
sodium octyl sulfate	Aldrich Chemical Co. (Milwaukee, WI.)
sodium phosphate, dibasic, anhydrous	Fisher Chemical (Fair Lawn, NJ)
sodium phosphate, monobasic	Fisher Chemical (Fair Lawn, NJ)
tri-n-octylamine	Sigma Chemical Co. (St. Louis, MO)
tris(hydroxymethyl)aminomethane (TRIS)	Fisher Chemical (Fair Lawn, NJ)
triton X-100	Fisher Chemical (Fair Lawn, NJ)
universal blocker	DAKO Corp. (Carpinteria, CA.)
zinc chloride	Fisher Chemical (Fair Lawn, NJ)
xylenes	Fisher Chemical (Fair Lawn, NJ)

2.1.2 Animals

All animal procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Alberta Health Sciences Animal Welfare Committee. Male Sprague-Dawley rats (200-300g) and male Mongolian gerbils (50-60g) were housed in pairs (separate rooms for rats and gerbils) and kept under controlled temperature (19-21ºC) and 12h light/12h dark conditions. The animals had free access to food and water. The gerbils, having originated out-of-province, were permitted to recover from shipment for 4 days before being used in experiments.

2.1.3 Drugs

PEH, FPEH, MePEH and MeOPEH (Fig. 2) were synthesized at the University of Alberta (Dr. E.E. Knaus, Faculty of Pharmacy and Pharmaceutical Sciences), dissolved in pure corn oil (Mazola brand) and administered intra-peritoneally.

2.1.4 Drug, Vehicle and Buffer Preparation

Weighing of experimental reagents was performed using a Mettler AE160 Balance (Mettler Instrumente, Switzerland). Vortex-assisted mixing was performed using a Sybron/Thermolyne MaxiMix vortex (Thermolyne Sybron Corp., USA). Final solutions of PEH and its analogues in corn oil (Mazola) were prepared on the same day on which they were to be used. The stock solutions of these drugs were synthesized by Dr. E.E. Knaus of the Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta (for synthetic details see Paslawski et al., 2001 and Sowa et al., 2005). Large volumes of buffer solutions were mixed using a Thermolyne Type 1000 stir plate (Thermolyne Sybron Corp., USA).

2.1.5 Animal Handling

Gerbils and rats were handled by a single handler, every 1-2 days following arrival at our animal facility, in order to increase familiarity with the principal handler and in an attempt to reduce stress associated with the manipulations involved in the experiments outlined in this thesis.

2.1.6 Isolation, Preservation and Storage of Animal Brains

Animals were decapitated at pre-determined times using a guillotine. After decapitation, the brains were immediately frozen by placing them into isopentane on solid carbon dioxide. The brains were then transferred to another receptacle and frozen at -80ºC in a Sanyo Ultra Low Termperature freezer (Sanyo Electric Co., Japan) until needed.

2.1.7 Preparation of Microscope Slides

Brains of gerbils used in neuroprotection experiments were thawed to -13 °C in a cryostat chamber after removal from their initial storage location (-80°C). The cryostat was then used in the sectioning of gerbil brains into 20 µm-thick sections and the sections were transferred to electrocharged microscope slides (Fisherbrand Super*frost*®/Plus, Fisher Scientific Co., USA). Slides were stored at -4 °C for later staining and microscopy. Microscopy was performed using a Nikon Labophot-2 microscope with 10X eyepiece and 1, 10, 20 and 40X objective lenses (Nikon Corp., Japan).

2.1.8 Measurement of pH

When pH determination was required, it was conducted using an Acumet 915 pH Meter (Fisher Scientific, USA) which was calibrated prior to use.

2.1.9 Preparation of Double-Distilled Water (ddH$_2$O)

Distilled water supplied in the Clinical Sciences Building (single-distilled) was then distilled a second time in our laboratory using a Corning Mega-Pure 3L automatic distilling apparatus (Corning, USA), yielding double-distilled water, which was then stored at room temperature in a sealed, glass receptacle. Prior to use, double-distilled water was filtered through a Fisherbrand mixed bed organic filter (Fisher Scientific, USA).

2.1.10 Glassware Preparation and Cleaning

All used glassware was rinsed in tap water and stored in a plastic glassware container prior to washing. All glassware was loaded into a Miele

67704 Lavabor industrial dishwasher (Miele Inc., USA) and subjected to two wash cycles with Fisherbrand Sparkleen chlorinated laboratory detergent (Fisher Scientific Co., USA) prior to two cycles of rinsing, the first with tap water and the second with single-distilled water. After the dishwasher step, the glassware was dried in a Precision 18EM mechanical convection oven (Precision Scientific, USA). Test tubes used in the radiochemical assays for MAO activity/inhibition were soaked in Contrad (DeCon Labs, USA) and subjected to 30 minutes of sonication using a Mettler Electronics Ultrasonic Cleaner (Mettler Electronics Corp., USA). The tubes were then transferred from the ultrasonic cleaner to the dishwasher for two cycles of washing with Sparkleen followed by two cycles of rinsing (tap and single-distilled water) and finally drying in a laboratory drying oven.

Test tubes used in HPLC experiments were scrubbed by hand using test tube brushes and Sparkleen, followed by thorough rinsing with single-distilled water prior to drying in the mechanical convection oven.

2.1.11 Statistical Analysis

Statistical analysis of all data was performed using GraphPad Prism® software (GraphPad Software, Inc., San Diego, California, USA). Unless otherwise stated, statistical analysis involved one-way analysis of variance (ANOVA) followed by Dunnet's t-Test or the Neuman-Keuls post test for significance versus controls and significance in multiple comparisons, respectively. Differences in the means of the data were considered to be significant if $p<0.05$.

Chapter 3

Neurochemistry of PEH Analogues: Acute Effects on Brain Amino Acid Levels

3.1. Introduction

Brain GABA and alanine levels increase markedly in gerbils and rats following administration of phenelzine (PLZ) or phenylethylidenehydrazine (PEH), a putative metabolite of PLZ (Popov and Matthies, 1969; Baker et al., 1991; McManus et al., 1992; Todd and Baker, 1995; Paslawski et al., 2001; Parent et al., 2000, 2002). Both compounds have been demonstrated previously to be neuroprotective in a gerbil model of transient forebrain ischemia (Todd et al., 1999; Tanay et al. 2002).

Following initial demonstration that FPEH, like PLZ and PEH, produced marked increases in brain levels of GABA and alanine in rats (Sowa et al., 2005), FPEH, MePEH and MeOPEH were examined more rigorously with respect to their effects on brain amino acids in the gerbils, the animal model to be used in the ischemia studies. The results of those studies are reported here.

3.2 Methods

3.2.1 Dose-Response and Time-Response Experiments

In dose-response studies, a PEH analogue (5, 7.5, 15 or 30mg/kg, i.p.) or vehicle (corn oil) was administered to the gerbils and the animals were returned to their cages, where they had free access to food and water. The animals were decapitated 4 hours after drug administration and their brains were removed and frozen (see Chapter 2 for details) for determination of enzyme activity and amino acid levels.

Animals were killed 4 hours post-injection because PEH and PLZ had previously been shown to have maximum effects on GABA, alanine and glutamine at 4-6 hours post-injection in rats (Paslawski et al., 1995; Baker et al., 1991; McManus et al., 2001; Tanay et al., 2001).

In time-response studies FPEH (30mg/kg, i.p.) or vehicle (corn oil) was administered to the gerbils and the animals were returned to their cages, where they had free access to food and water. The animals were decapitated 1, 3, 4, 6, 24 or 72 hours after drug administration and their brains were removed and frozen for later determination of enzyme activity and amino acid levels.

3.2.2 Determination of Brain Amino Acid Levels

Brains isolated from animals given a single dose of drug (PEH, PLZ or PEH analogue) or vehicle, as outlined in *General Methods*, were homogenized in 5 volumes of ice-cold double-distilled water using a Tri-R Stir-R S36C variable speed homogenizer (Tri-R Instruments, Inc., Rockville Centre, NY). All aliquots were kept on ice unless otherwise stated. Portions of the homogenate were diluted an additional 5-fold in 100% methanol in microfuge tubes and the remaining homogenates were frozen for future use. Methanol-diluted aliquots were re-homogenized by hand using a Kontes Pellet Pestle Motor hand homogenizer (Kimble/Kontes, USA) and then allowed to sit on ice for 10 minutes prior to centrifugation at 4°C in an Allegra 21R centrifuge (Beckman Coulter, Germany). Supernatants were acquired, diluted 10-fold in ice cold double-distilled water, and centrifuged again. Supernatants from these aliquots were then analyzed by HPLC. Prior to chromatographic separation, amino acids were chemically derivatized to fluorescent isoindole compounds by reaction with o-phthalaldehyde in alkaline medium, with 2-mercaptoethanol serving as the reducing agent (Fig. 4; 5 µL of fluoraldehyde reagent containing o-phthaldialdehyde and mercaptoethanol were added to 5 µL of sample; the sample was then held in the injection loop of the solvent management system for 90 seconds prior to injection). The amino acid-containing samples were held at 4°C prior to derivatization. Chromatographic separation was achieved using a Waters Alliance 2695 separations module coupled with a Waters µBondapak C18 pre-column and Waters Novapak column (C18, 5µm, 3.9x150mm) all held at 30°C. The mobile phase mixture was pumped at 0.5 mL/minute. Mobile phase "A" consisted of 0.08M NaH_2PO_4, 240 mL MeOH, 20 mL CH_3N and 10 mL tetrahydrofuran in 900 mL distilled water, adjusted to pH 6.2 with 10N NaOH. Mobile phase "B" consisted of 0.04M NaH_2PO_4, 1.11 L MeOH and 60 mL tetrahydrofuran in 1.34 L distilled water, with pH adjusted to 6.2 using 10N NaOH. Both mobile phases were filtered and degassed under vacuum using 0.2 µm filters. Initial mobile phase composition was 60% mobile phase "A" and 40% mobile phase "B" and this composition was gradually adjusted until it reached 100% mobile phase "B" at 15 minutes. Total run time was 45 minutes per sample. Fluorescence emitted by the thioalkyl derivatives of the brain amino acids was detected using a Shimadzu RF10A fluorescence detector (260nm excitation and 455 nm emission wavelengths). Chromatographic data were analyzed using Empower software. A calibration curve consisting of known, varying amounts of the amino acids of interest was carried through in parallel with each assay run (Fig. 5).

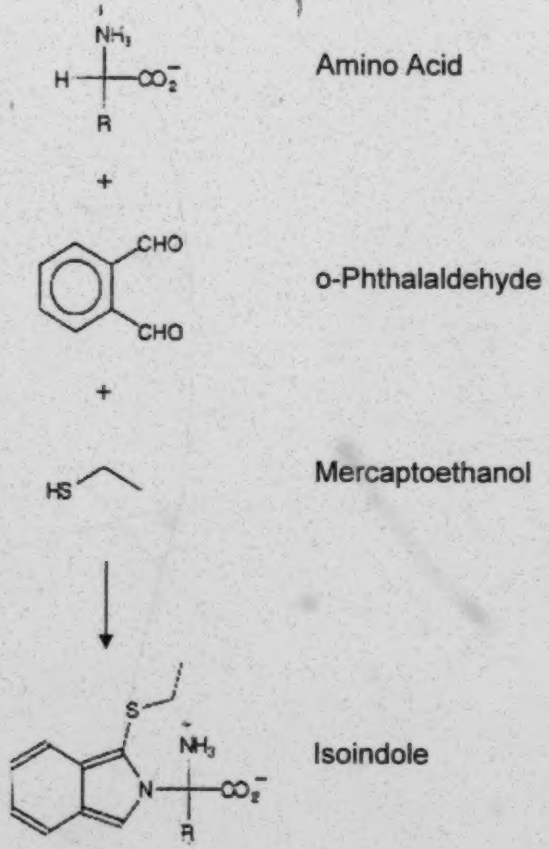
Amino Acid
o-Phthalaldehyde
Mercaptoethanol
Isoindole
NH3
H
CO2
R
CHO
CHO
HS
S
N

Figure 4. Schematic representation of the derivatization of an amino acid with o-phthalaldehyde.

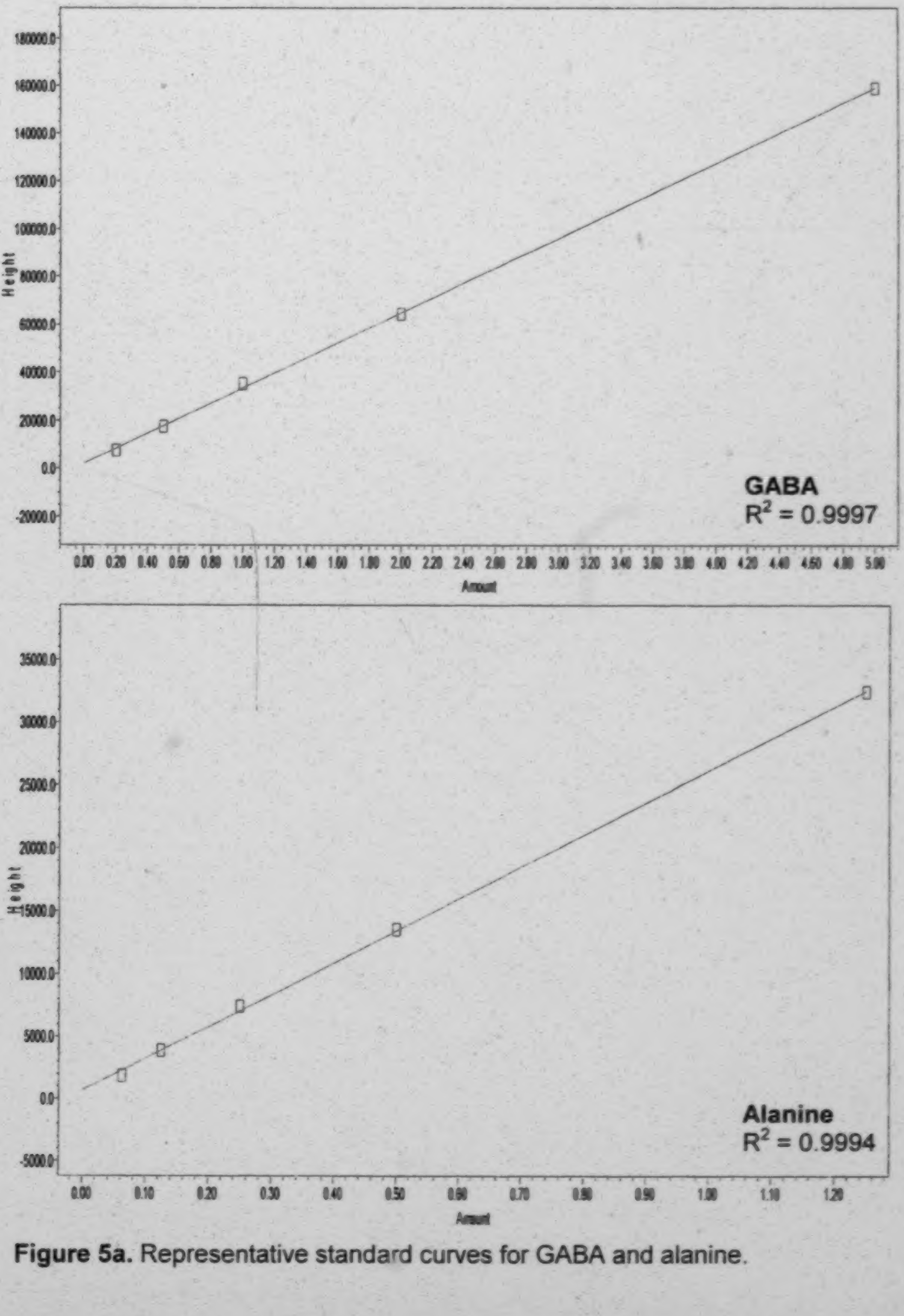

Figure 5a. Representative standard curves for GABA and alanine.

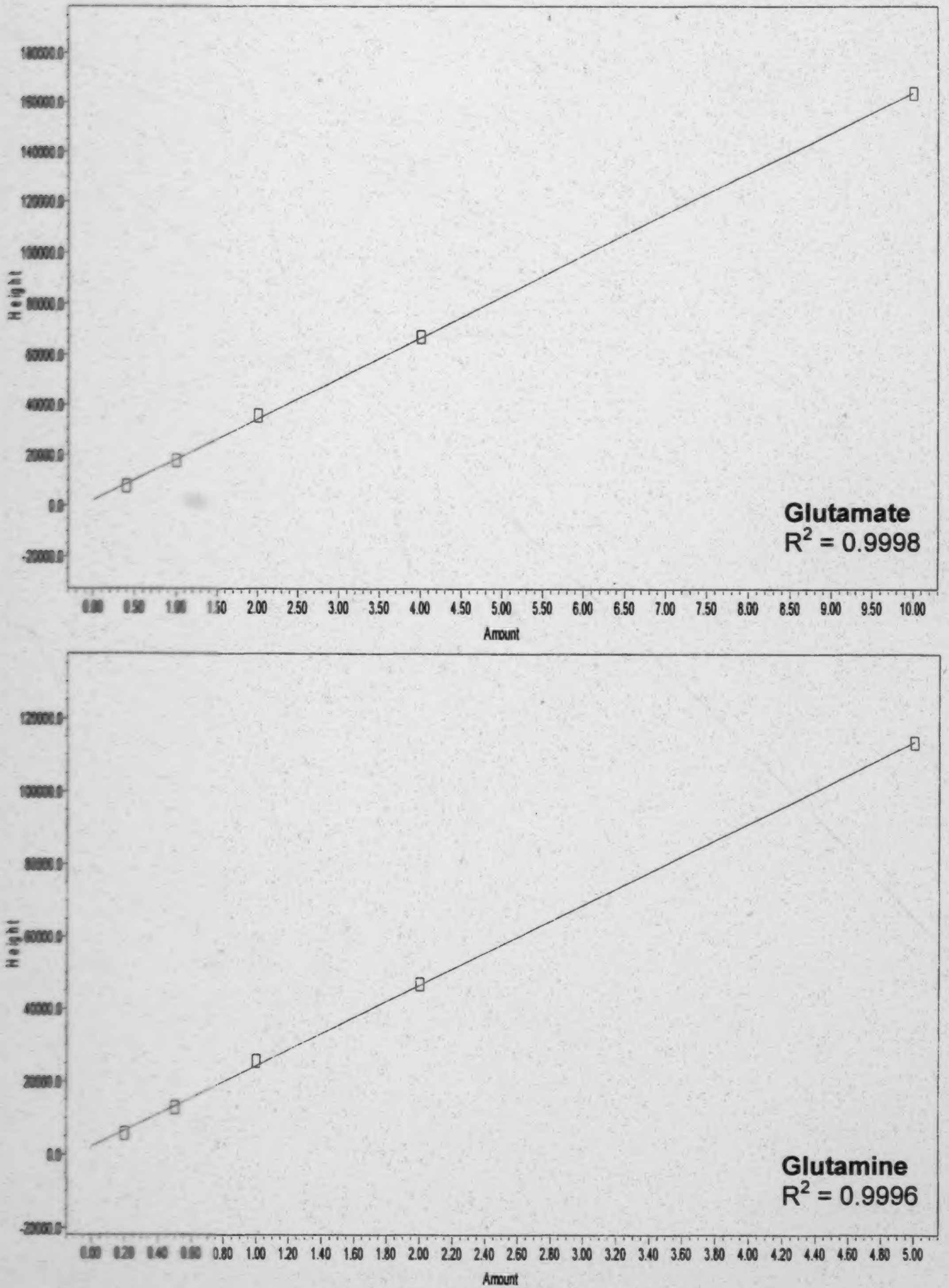

Figure 5b. Representative standard curves for glutamate and glutamine.

3.3 Results

3.3.1 Dose-Dependence of PEH Analogue-Associated Changes in Levels of Brain Amino Acids

All of the PEH analogues dose-dependently increased brain GABA and alanine content (Fig. 6), but only FPEH decreased brain glutamine levels (Fig. 7). FPEH also displayed a complex effect on brain glutamate, with reductions noted at 5, 7.5 and 15 mg/kg but not at 30 mg/kg (Fig. 7), while MePEH and MeOPEH had no significant effects on brain levels of glutamate or glutamine (Fig. 7). When the time study in the gerbils was extended with FPEH (30 mg/kg), elevated GABA and alanine levels were still evident at 24 hours after FPEH administration (Fig. 8) while glutamine levels returned to normal between 6 and 24 hours (Fig. 9).

3.4. Discussion

Examination of the effects of PEH analogues on gerbil brain amino acid levels revealed dose-dependent effects of all three analogues on GABA and alanine levels (Figs. 6 and 7). After the initial studies on brain GABA were conducted and showed a marked elevation of GABA at a dose of 30 mg/kg (Fig. 6), similar to what had been observed previously with PEH (Todd et al., 1999; Tanay et al., 2002) this dose was chosen for the neuroprotection screen in the cerebral ischemia model (see Chapter 7) since the same dose of PEH had been shown to be neuroprotective in this model (Todd et al., 1999; Tanay et al., 2002). Since FPEH was the only one of the analogues to display a significant neuroprotective effect in these studies (Chapter 5), examination of FPEH was extended to include a time-response study (Figures 8 and 9). The increases in brain levels of GABA and alanine (seen with all of the drugs at 4 hours after administration) and the transient decrease in brain glutamine levels (seen with FPEH; Figs. 7 and 9) are consistent with what has been observed previously with PLZ and PEH in rats (Paslawski et al., 1995; 2001; Baker et al., 1991).

The effect of FPEH on glutamate levels is difficult to explain. It is possible that at the lower doses, drug-associated inhibition of GABA-T (to be discussed in following chapters) leads to a reduction in glutamate formation from α-KG and an overall decrease in brain glutamate levels. At the highest dose of FPEH, glutamate levels may rise to control values as a consequence of several factors including: (1) conversion of excess alanine to pyruvate, which may subsequently be converted to glutamate; (2) inhibition of glutamine synthetase by alanine, preventing the conversion of glutamate to glutamine and subsequently causing accumulation of glutamate; and (3) feedback inhibition of GAD by GABA (which has accumulated due to drug-associated inhibition of GABA-T), preventing glutamate from being converted to GABA. It is of interest that Costa et al. (2004) demonstrated, using cortical slices *in vitro*, a bell-shaped relationship between recovery of neuronal electrical activity following oxygen-glucose deprivation and dose of vigabatrin (an inhibitor of GABA-T). In their study, GABA itself and the GABA transport inhibitor, tiagabine, both displayed bell-shaped relationships between their concentrations and their neuroprotective effects.

It is also of interest that glutamine levels are decreased below control levels by FPEH at all doses studied (Fig. 7). The decreases may be a result of reduced availability of glutamate for conversion to glutamine via glutamine synthetase, since 5, 7.5 and 15 mg/kg FPEH also reduced brain glutamate levels, but other as yet unknown factors are likely involved since (1) in the time study on FPEH (Fig. 9), the decrease in glutamine is longer-lasting than the decrease in glutamate and (2) the 30 mg/kg dose of FPEH was not associated with a reduction of brain glutamate levels.

While much of the focus with GABAergic drugs has been on brain GABA levels, the elevation of both GABA and alanine levels by PEH and its analogues necessitates consideration of the possible (patho-)physiological effects of changes in brain alanine levels. Inhibition of ala-T and the consequent elevation of brain alanine levels that occurs may be undesired activities in the setting of cerebral ischemia and may not be benign with respect to their effects on neuronal survival and the position of the balance between neurorescue and neurotoxicity.

Alanine-mediated inhibition of glutamine synthetase leads to accumulation of ammonia and potentially to reversal of plasma membrane transporters for alanine, glutamate and GABA (Laake et al., 1995). Specific inhibition of glutamine synthetase using L-methionine sulfoximine has been demonstrated to result in a reduction in astrocytic glutamine and an increase in astrocytic glutamate (Laake et al., 1995). Additionally, ala-T is a major contributor to the catabolism of glutamate and, as such, can participate in protecting neurons against glutamate-mediated excitotoxicity (Matthews et al., 2000). It is possible, then, that the potentially neuroprotective/neurorescue GABA-elevating effect of

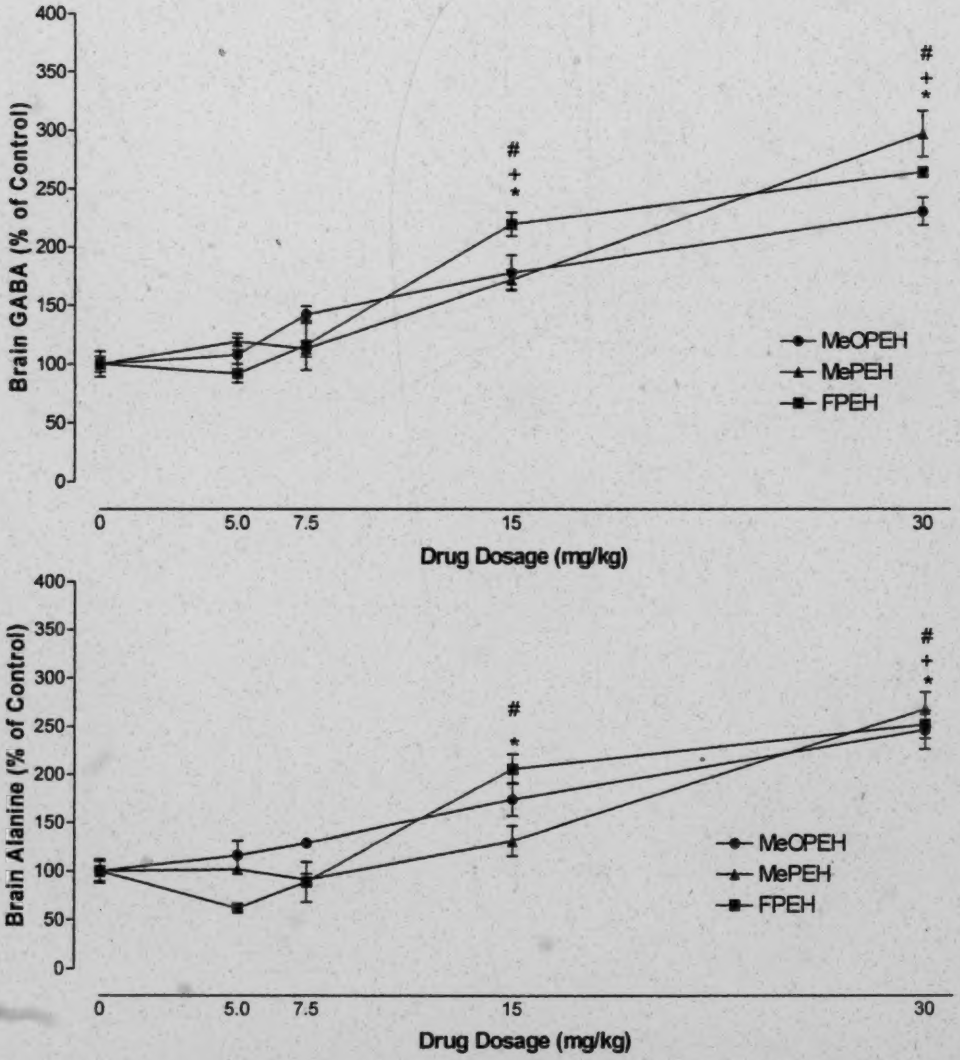

Figure 6. Dose-dependent effects of PEH analogues on gerbil brain GABA and alanine levels 4 hours post-administration (30 mg/kg i.p.). Results are expressed as means ± S.E.M. (N=4-6 animals per group). Control brain levels of GABA and alanine were 335 ± 44 µg/g and 52 ± 11 µg/g, respectively. *, + and # indicate $p < 0.05$ vs. controls for MeOPEH, MePEH and FPEH, respectively.

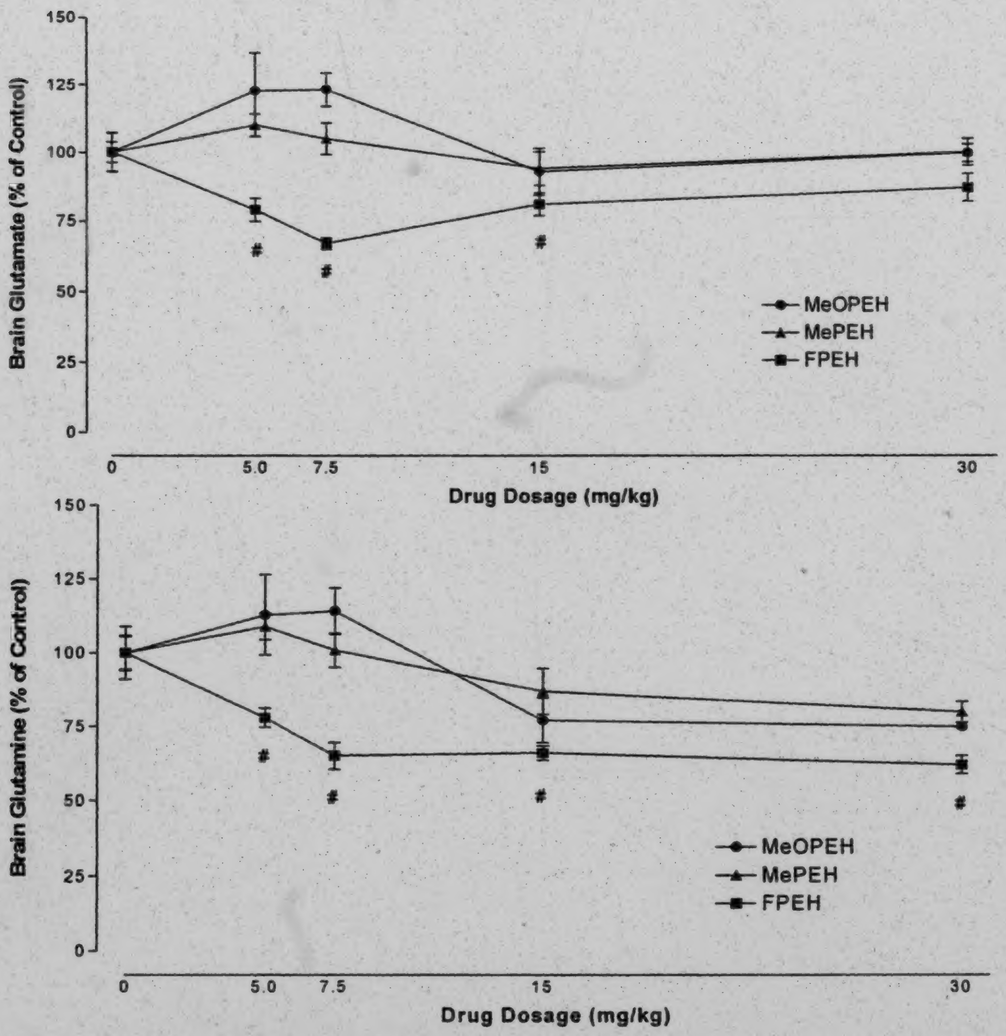

Figure 7. Dose-dependent effects of PEH analogues on gerbil brain glutamate and glutamine levels 4 hours post-administration (30 mg/kg i.p.). Results are expressed as means ± S.E.M. (N=4-6 animals per group). Control brain levels of glutamate and glutamine were 1831 ± 227 µg/g and 960 ± 275 µg/g, respectively. *, + and # indicate $p < 0.05$ vs. controls for MeOPEH, MePEH and FPEH, respectively. Where standard error bars are smaller than the suze of the symbol, they are not shown.

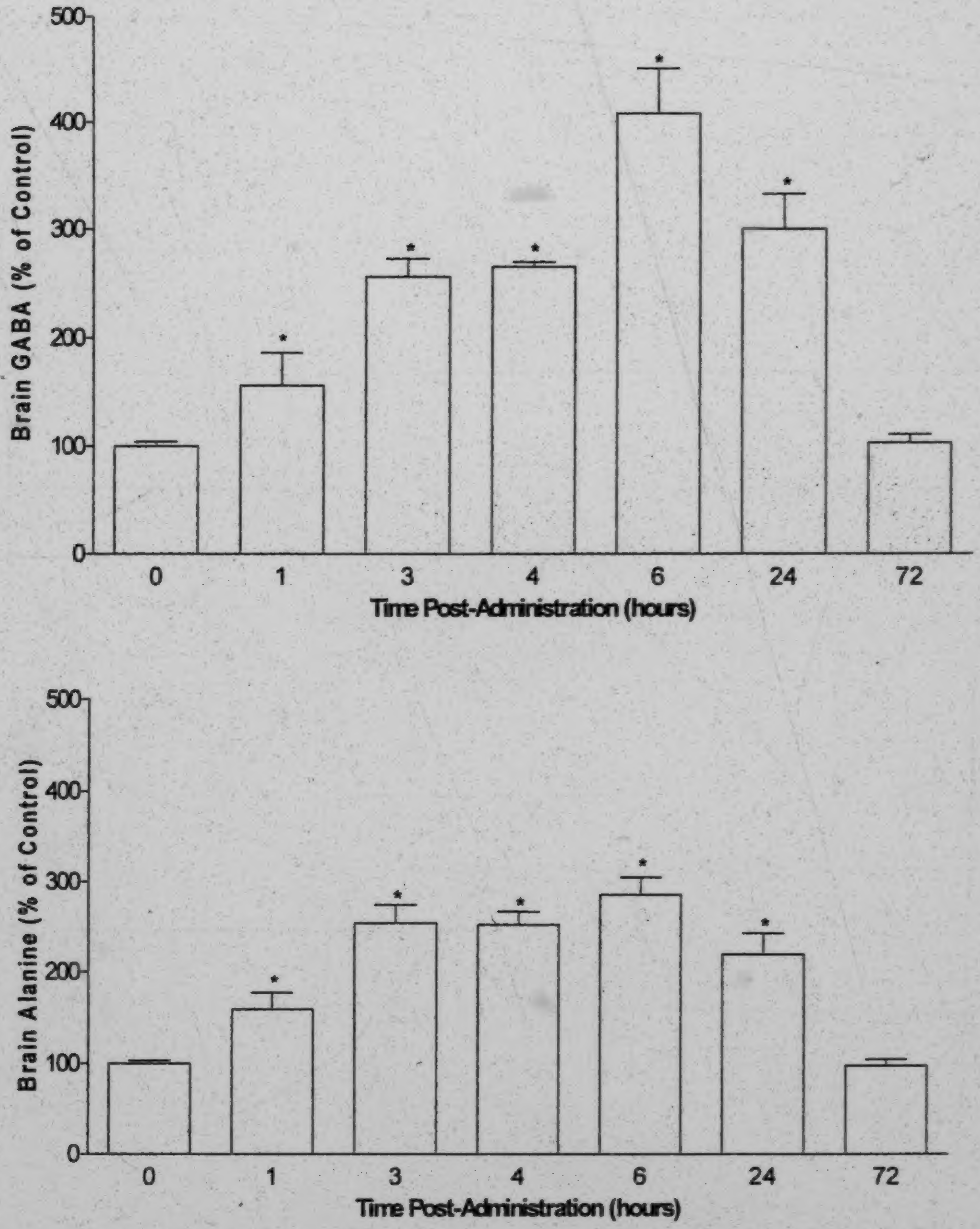

Figure 8. Time-dependent effects of FPEH (30 mg/kg) on brain GABA and alanine levels in gerbils. * signifies $p < 0.05$ vs. control animals. Results are expressed as means ± S.E.M. (N=4-8 animals per group).

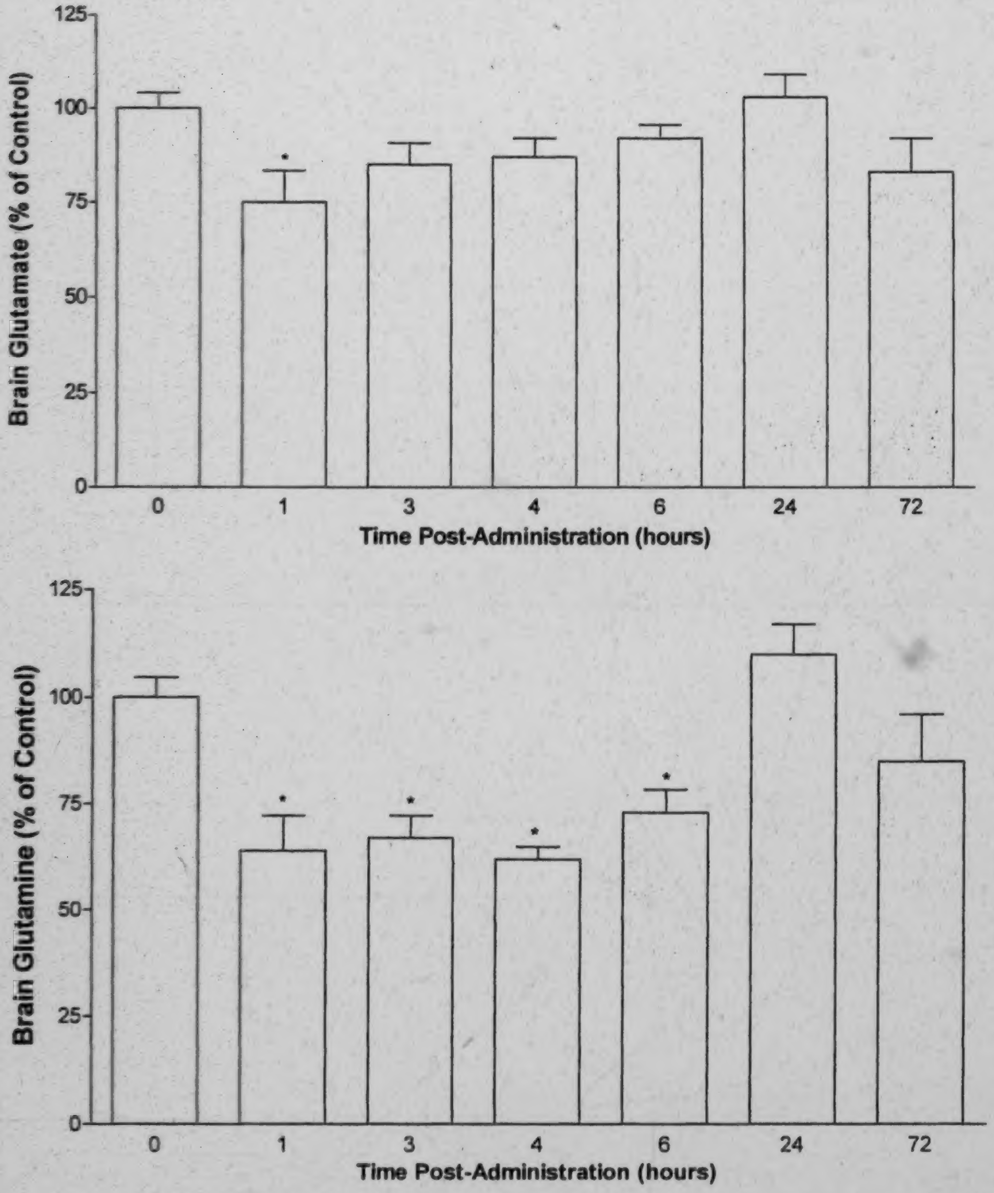

Figure 9. Time-dependent effects of FPEH (30 mg/kg) on brain glutamate and glutamine levels in gerbils. * signifies $p < 0.05$ vs. control animals. Results are expressed as means ± S.E.M. (N=4-8 animals per group).

FPEH is at least partially offset by its inhibition of ala-T (thereby reducing the catabolism of both glutamate and alanine) and the subsequent elevation of brain alanine levels (the consequences of which may include inhibition of glutamine synthetase and accumulation of ammonia). Inhibition of glutamine synthetase by elevated alanine or by FPEH directly may also account for the decrease in brain glutamine, but this remains to be investigated. While increases in brain alanine thus may have had important consequences in the short term effects of the PEH analogues, it is of interest that no increase in alanine levels was observed in frontal cortex tissue from gerbils treated for 7 days with the drugs (see Chapter 5); in contrast an increase of GABA levels was still evident.

In summary, all three PEH analogues were demonstrated in this acute study to dose-dependently elevate brain GABA and alanine levels in Mongolian gerbils. FPEH was the only one of the three to cause a decrease in glutamate levels, and a subsequent time study showed that the glutamate and glutamine levels returned to control values much more rapidly than did the GABA and alanine levels.

3.5 References

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Chapter 4

Neurochemistry of PEH Analogues: Effects on Enzyme Activity

4.1. Introduction

As was discussed earlier, brain GABA levels increase in rats and gerbils following administration of PLZ (Baker et al., 1991; McManus et al., 1992), PEH (Paslawski et al., 2001; Parent et al., 2002) or analogues of PEH under investigation here (Chapter 3). Previous studies of PEH and PLZ in rats showed inhibition of both GABA-T and ala-T by those drugs; the inhibitory effects contributed to the GABA and alanine-elevating effects of PEH and PLZ (McManus et al., 1992; Todd and Baker, 1995; Paslawski et al., 2001; Tanay et al., 2001) and, given the effects of the three PEH analogues on GABA and alanine levels, it was predicted that they would also inhibit these enzymes in brain. Hydrazine-containing compounds are thought to interfere with the activity of pyridoxal phosphate (PLP)-dependent enzymes by reducing the availability of PLP for binding to the enzymes. While the PEH analogues differ from PEH because of substitution at the para position of the phenyl ring, the analogues still possess the hydrazine moiety and should, therefore, retain hydrazine-associated biological activities.

Pyridoxal phosphate (PLP), a product of the hepatic metabolism of Vitamin B_6 (also known as pyridoxine), is a cofactor for several enzymes, including GABA-T and ala-T, and is involved in processes including red blood cell generation, antibody production, volume regulation, fat and carbohydrate metabolism and amino acid metabolism (Tunnicliff and Ngo, 1998; Holt et al., 2004). PLP attaches to the active site of an enzyme by forming a Schiff base with the ε-amino group of a lysine within the enzyme's active site; the resultant

Schiff base complex is also referred to as an *internal* aldimine (Tunnicliff and Ngo, 1998), formed by a reaction involving the aldehyde group of the pyridoxal phosphate moiety and an amino group from the lysine moiety. Amino acids undergoing transamination take the place of the active site lysine with respect to their interaction with the pyridoxal phosphate molecule – i.e. the amino acid undergoing transamination forms a Schiff base complex with the pyridoxal phosphate molecule (a complex referred to as an *external* aldimine), in place of the amine group of the lysine at the enzyme's active site, thereby temporarily removing the PLP from the active site of the enzyme. Hydrazides appear to react with PLP, reducing the availability of PLP to act as a cofactor in enzyme-catalyzed reactions (Tunnicliff and Ngo, 1998), although the exact mechanism by which this occurs has not been fully characterized as of yet.

The interaction between hydrazides and PLP may occur in solution, but slow-binding inhibition of the GABA-T enzyme, itself, by hydrazides has also been described (Lightcap and Silverman, 1996). Based on what is currently suggested regarding the mechanism of enzyme inhibition by the hydrazides, it was hypothesized that the activity of PLP-dependent enzymes such as GABA-T and ala-T would be reduced by the PEH analogues.

In this chapter, I report the results of experiments examining the effects of PEH analogues on the activities of GABA-T and ala-T both *ex vivo* (i.e. brain tissue removed from gerbils administered the analogues via the i.p. route is assayed for enzyme activity) and *in vitro*. Preliminary studies on the interactions of PEH and its analogues with cytochrome P450 (CYP) enzymes were also conducted. CYP enzymes catalyze the metabolism of a large number of drugs, and competition for these enzymes can lead to serious drug-drug pharmacokinetic interactions. Screening for interactions with CYP enzymes is now an integral part of drug development, and such a screen was included in this research project.

Dose and time studies were conducted on the effects of FPEH on inhibition of gerbil brain MAO-A and –B *ex vivo*, although similar studies in rat brain by Paslawski et al. (2001) indicated that PEH had only weak inhibitory effects on MAO compared to PLZ.

4.2 Methods

4.2.1 Ex Vivo Determination of GABA-T and Ala-T Activity

Gerbil brains removed from animals to which PEH analogues had been administered were homogenized in five volumes of ice-cold homogenizing medium using a Tri-R Stir-R S36C variable speed homogenizer (Tri-R Instruments, Inc., Rockville Centre, NY). Homogenates to be used in GABA-T and ala-T assays were then further diluted in 4 volumes or 2 volumes of homogenizing medium, respectively. To microfuge tubes on ice were then added 10 μL of tissue homogenate (blanks received 10 μL of ddH_2O instead of tissue homogenate) and 20 μL of incubation medium. The microfuge tubes were then incubated at 37°C for 30 minutes (GABA-T assay) or 60 minutes (ala-T assay). The reaction was terminated by placing the microfuge tubes on ice and adding 100 μL of previously-prepared (see below), ice-cold tri-n-octylamine (TOA), a liquid anion exchanger. Tubes were then capped, vortexed and centrifuged for 4 minutes at 15,000 x g. Following centrifugation, 35 μL of the organic layer was drawn off and added to counting vials containing 4 mL of scintillation fluid (Beckman Coulter ReadySafe™ liquid scintillation cocktail) and radioactivity was counted using a liquid scintillation counter (Beckman LS 6000SC).

The TOA was prepared prior to the experiment as follows: 2.2 mL of TOA was added to 22.8 mL of ethyl acetate and to the resultant solution was added 49.2mL of double-distilled water to which had been added 850 μL of phosphoric acid. The resultant mixture was then shaken for 4 minutes and then left to sit overnight at 4°C in order to allow for clear discrimination of the organic and aqueous phases. The top (organic) layer was transferred into a separate container and the bottom (aqueous) layer was discarded.

The incubation medium for GABA-T and ala-T assays was composed of: 1 μL of ^{3}H-GABA (1 μCi; GABA-T assay) or 1 μL of ^{3}H-alanine (1 μCi; ala-T assay), 248 mM Tris, 5 mM non-radiolabeled GABA or ala, 5 mM α-ketoglutarate, 1 mM NAD (GABA-T assay) or 1 mM NADH (ala-T assay) and 1 mM aminoethylisothiouronium bromide in double-distilled water. The composition of the homogenizing medium was as follows: 20% volume/volume glycerol, 1% volume/volume triton X-100, 100 μM glycerol, 1 μM pyridoxal phosphate, 1 mM sodium ethylenediaminetetraacetic acid and 5 mM K_2HPO_4 in double-distilled water, with a pH of 7.4. Adjustment of the pH of the homogenizing medium to 7.4 was accomplished using glacial acetic acid. This procedure has been described previously by McManus et al. (1992) and is a modification of the method of Sterri and Fonnum (1978).

4.2.2 In Vitro Determination of GABA-T and Ala-T Activity

In vitro determination of GABA-T and ala-T activity was conducted in a similar manner to that described in section 4.2.1, but with the following changes: (1) tissue homogenates were prepared from the brains of drug-naïve gerbils; (2) the source of drug in the tissue homogenates was exogenous addition of known amounts of PEH or its analogues to the microfuge tubes, generating solutions with known concentrations of the drugs. Seven to eight concentrations of each drug were studied, with an N of 5 or 6 for each concentration point. The drugs were taken up in DMSO prior to dilution with double-distilled water to make the appropriate concentrations.

4.2.3 Ex Vivo Determination of MAO Activity

Whole brain samples were analyzed using the procedure of Lyles and Callingham (1982), with radiolabelled 5-hydroxytryptamine and β-phenylethylamine as substrates for MAO-A and MAO-B, respectively. Tissue homogenates and substrates were prepared in 0.2 M potassium phosphate buffer, pH 7.8.

The radiolabelled substrates used were 5-hydroxy[G-^{3}H]tryptamine creatinine sulfate and 2-phenyl[1-^{14}C]ethylamine hydrochloride. Stock radiolabelled substrates obtained from suppliers possessed high specific radioactivity (e.g. [3H]5-HT, 15 mCi/μmol; [^{14}C]PEA, 43.7 μCi/μmol). Since only 1 μCi/μmol specific activity is required for good resolution in the assay procedure described here (Dr. A. Holt, personal communication), stock amine substrates were diluted with their respective unlabelled amines to give 5-HT at 50 mM, 1 μCi/μmol and PEA at 10 mM, 1 μCi/μmol.

Brains were homogenized in 80 vols of buffer and assays were carried out in triplicate in borosilicate glass culture tubes. Tubes were placed on ice, and to each were added 50 μl of tissue homogenate and 50 μl of appropriate radiolabelled substrate (diluted 1/100 from diluted stock). To blank tubes were added 10 μl of HCl. Samples were flushed, stoppered quickly and incubated in a water both at 37° for 10 minutes. The tubes were then placed on ice, and 10 μl HCl was added to each of the tubes except for the blanks. Ethyl acetate/toluene (1:1 v/v, water-saturated) (1 ml) was added to each sample. The tubes were vortexed briefly and centrifuged at low speed (1600 rpm) for 30 seconds. A portion (700 μl) of the upper (organic) layer was pipetted into a small scintillation vial. ReadySafe™ scintillation fluid (4 ml) was added to each scintillation vial, and the samples were counted for 3 minutes on a scintillation counter.

4.2.4 CYP Isozyme Inhibition Assay

Assays were conducted in black 96-well polystyrene microwell plates. Test compound stock solutions were prepared in 10% (v/v) DMSO. Human hepatic CYP enzyme isoforms were obtained from Gentest (Woburn, Mass.). The source of the enzymes is human cDNA expressed using a baculovirus (*Autographa californica*) expression system in which cells from the moth *Trichoplusia ni* (BTI-Tn-5B1-4) were used to produce the microsomes. Glucose-6-phosphate dehydrogenase was obtained from Cedarlane Laboratories, Ltd. (Ontario, Canada). An NADPH-generating solution was added to all wells. The composition of the NADPH-generating solution was: 0.83 mM $MgCl_2$, 0.83 mM glucose-6-phosphate, 16 µM NADP, 0.4 U/mL glucose-6-phosphate dehydrogenase and 5 mM KH_2PO_4 in double-distilled water. The test compound was then added to test wells while vehicle was added to control and blank wells. All test plates were then pre-incubated at 37°C for 10 minutes prior to the addition of pre-warmed CYP isozyme and fluorogenic substrate to all wells with the exception of the blanks. Plates were then incubated at 37°C for durations that varied with CYP enzyme being investigated (15 minutes for CYP1A2, and 30 minutes for CYP2B6/2C19/2D6/3A4). CYP isozyme-mediated metabolism was terminated by removal of plates onto ice followed by addition of a Tris/acetonitrile stop solution (1 volume of 0.5 M Tris added to 4 volumes of acetonitrile). Fluorescence readings were then made within 20 minutes, allowing time for the plates to cool to room temperature. The names and final concentrations of the fluorogenic substrates for the CYP isozymes are as follows: 3-cyano-7-ethoxycoumarin (CEC; 4.89 µM) for CYP1A2/2C19, 7-ethoxy-4-(trifluoromethyl)-coumarin (EFC; 2.45 µM) for CYP2B6, 3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-hydroxy-4-methylcoumarin (AMMC; 1.46 µM) for CYP2D6 and 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC; 48.8 µM) for CYP3A4. CYP1A2 and CYP2C19 dealkylate CEC to 3-cyano-7-hydroxycoumarin (CHC). The product of CYP2B6-mediated dealkylation of EFC and of CYP3A4-mediated dealkylation of BFC is 7-hydroxy-4-trifluoromethylcoumarin (HFC). The final product of CYP2D6-mediated dealkylation of AMMC is 3-[2-(N,N-diethylamino)ethyl]-7-hydroxy-4-

methylcoumarin hydrochloride (AHMC). Excitation and emission wavelengths (ex/em, measured in nm) for the products are 410ex/460em (CHC), 409ex/530em nm (HFC), and 390ex/460em (AHMC). The original concentrations and catalytic activities of the CYP enzymes are as follows (format used is *enzyme/original concentration/catalytic activity*): CYP1A2/1μM/1200 pmole/mg*min, CYP2B6/1μM/340 pmole/mg*min, CYP2C19/1μM/900 pmole/mg*min, CYP2D6/1μM/9200 pmole/mg*min, CYP3A4/1μM/2200 pmole/mg*min.

The Cheng-Prusoff equation [$K_i = (IC_{50}) / (1+(S/K_m))$] was used to convert the IC_{50} values obtained in these experiments into K_i values. In the Cheng-Prusoff equation, S and K_m represent the CYP enzyme substrate concentration and CYP enzyme-specific Michaelis constant, respectively. Fitting of curves to data obtained in these experiments was performed using GraphPad Prism® software. Sigmoidal dose-response curves were fitted to data, with the following constraints: maximum Y-value = 100%; minimum Y-value = 0%. The equation used is as follows: Y = Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope)) where X is the logarithm of concentration and Y is the response (% activity).

4.3 Results

4.3.1. Dose-Dependence of Drug-Associated Inhibition of GABA-T and Ala-T

Administration of PEH analogues resulted in dose-dependent inhibition of GABA-T (Fig. 10), with GABA-T activity being reduced to 61%, 60% and 68% of control levels at 4 hours following single, 30mg/kg i.p. doses of FPEH, MePEH and MeOPEH, respectively. The effects of the PEH analogues on ala-T activity were more pronounced, with the activity of that enzyme being reduced to 42%, 45% and 57% of control levels by 30mg/kg FPEH, MePEH and MeOPEH, respectively.

4.3.2. Time-Dependence of FPEH-Associated Inhibition of GABA-T and Ala-T in Mongolian Gerbils

Administration of FPEH was associated with a time-dependent reduction in the activity of brain GABA-T. The effect was greatest at 4 hours post-administration (GABA-T activity reduced to 61% of control) and was persistent, as it was still present at 72 hours following a single administration of 30mg/kg FPEH (GABA-T activity reduced to 81% of control level) (Fig. 11).

The pattern of the effect of FPEH administration on ala-T activity was similar to that seen on GABA-T activity (Fig. 11). Ala-T activity was lowest 4 hours post-administration (42% of control) and the effect was persistent, with ala-T activity recovering only to 81% of control levels at 72 hours post-administration.

4.3.3. Concentration-Effect Relationships (In Vitro)

Both FPEH and PEH (Figures 12 and 13) dose-dependently inhibited GABA-T and ala-T *in vitro*.

4.3.4 Effects of FPEH on MAO-A and MAO-B

The results of dose- and time-response studies with FPEH are shown in Figures 14 and 15. Inhibition of MAO-A or B did not occur until the 30 mg/kg dose, and even then inhibition was only 25% for MAO-A and MAO-B. In contrast, PLZ at 30 mg/kg inhibited MAO-A completely and MAO-B by more than 90%.

4.3.5 Effects of PEH and PEH Analogues on Cytochrome P450 Isozyme-Mediated Metabolism

PEH and the PEH analogues differentially interfered with CYP enzyme-mediated metabolism of fluorogenic substrates. Concentration vs. effect curves for these interactions can be seen in Figs. 16 through 20 and the K_i values for these activities can be seen in Table 2. In general, the drugs displayed their least potent effects on CYP2D6 and CYP3A4 and were most potent with respect to inhibition of CYP1A2-, CYP2B6- and CYP2C19-mediated metabolism (Table 2).

4.4. Discussion

GABA has been implicated in the treatment or pathophysiology of several psychiatric disorders, including affective disorders (Brambilla et al., 2003), epilepsy (Czuczwar and Patsalos, 2001), panic disorder (Johnson et al., 1994), and schizophrenia (Wassef et al., 2003; Costa et al., 2004; Coyle, 2004). Drugs such as benzodiazepines potentiate GABA-stimulated $GABA_A$ receptor activity, agents such as tiagabine reduce the reuptake of GABA, and others such as vigabatrin and PLZ decrease the metabolic breakdown of GABA. Major disadvantages of many drugs used in treating medical conditions include their wide range of pharmacologic activities, some of which may cause undesired or complicating side effects (inhibition of MAO as well as GABA-T and ala-T by

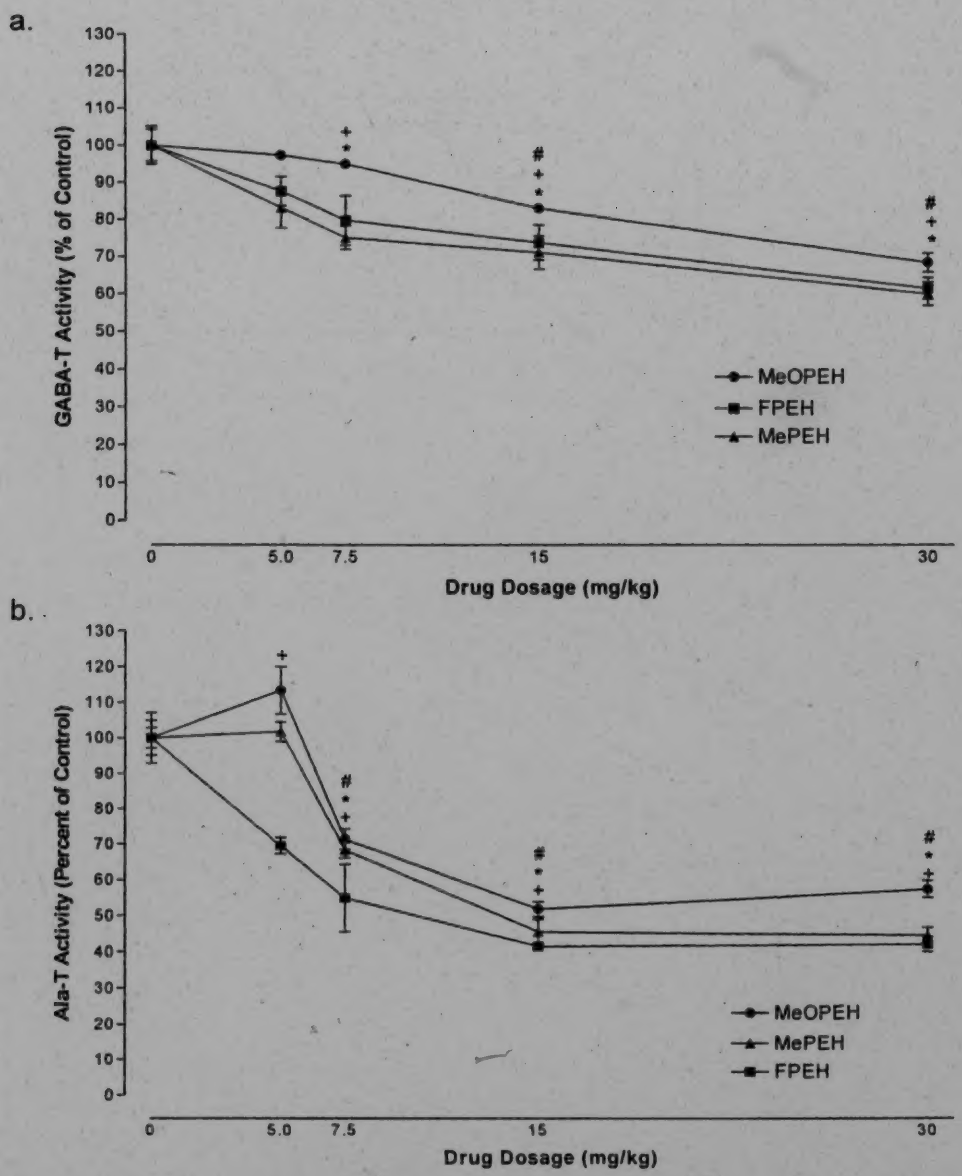


Figure 10. Dose-dependent effects of PEH analogues on the activities of GABA-T (a) and ala-T (b) 4h after drug administration. Results are expressed as means ± S.E.M. (N = 4-6 animals). *, + and # indicate $p < 0.05$ vs. controls for MePEH, FPEH and MeOPEH, respectively.

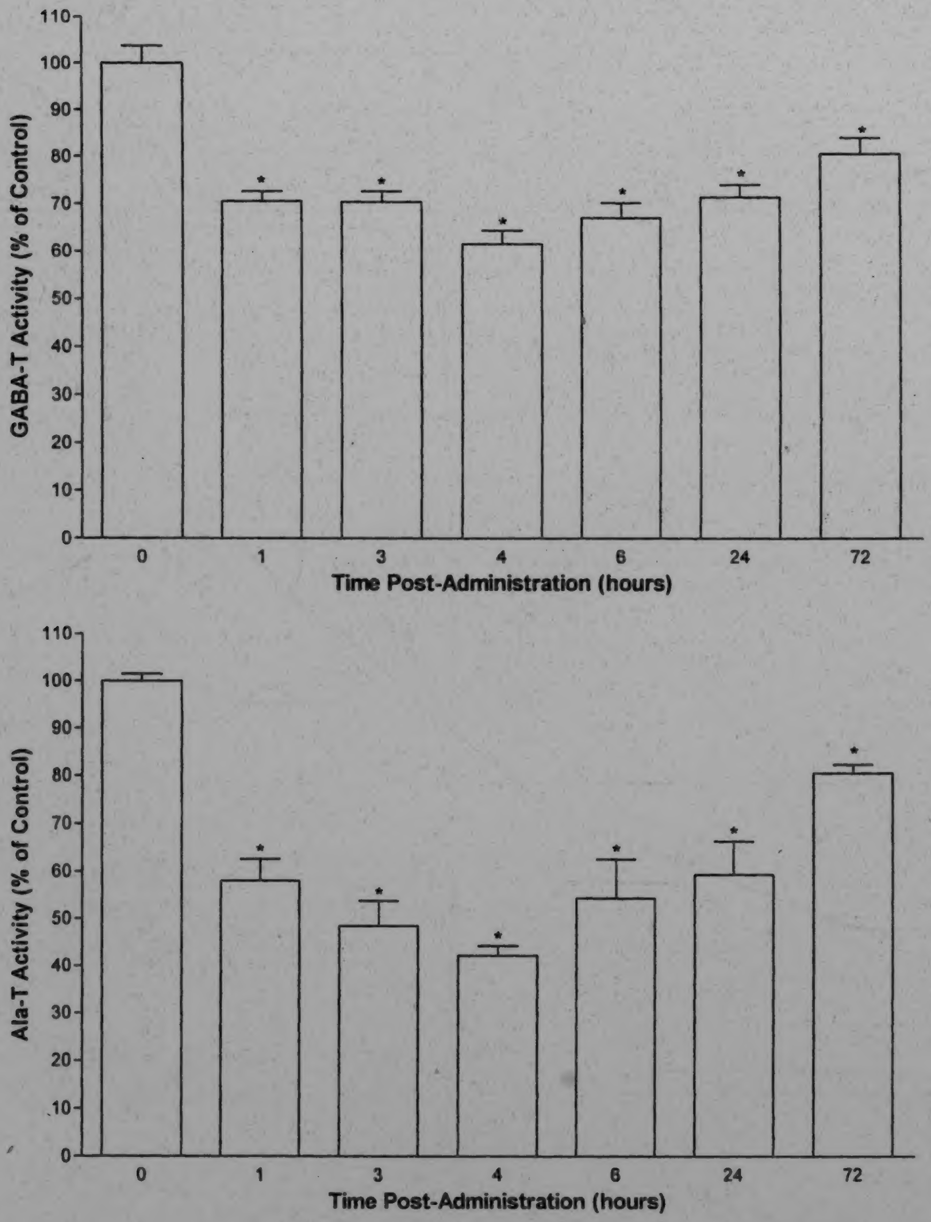
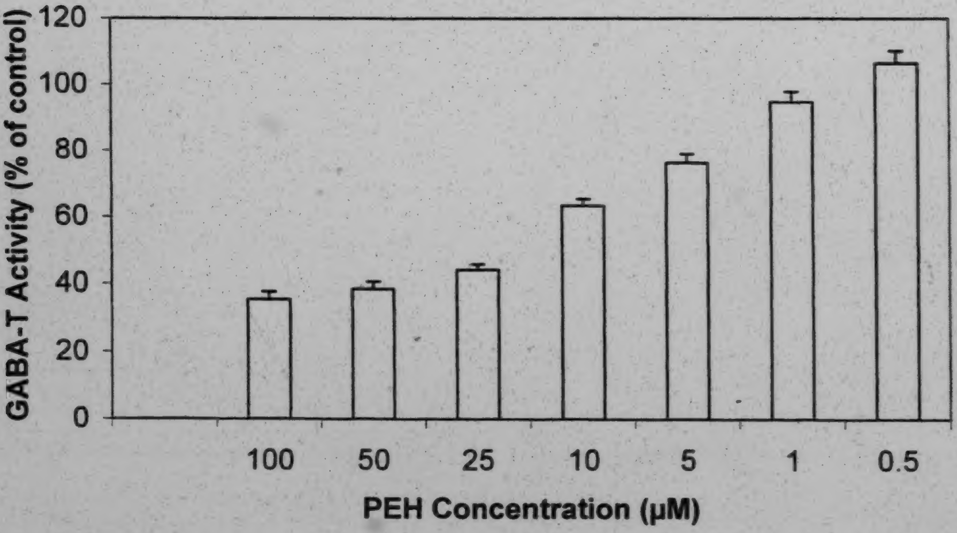


Figure 11. Inhibition of GABA-T and ala-T (% of control, mean ± SEM) following a single injection of FPEH (30 mg/kg, i.p.). * indicates $p < 0.05$ vs. control group. n = 4-8 animals per group.



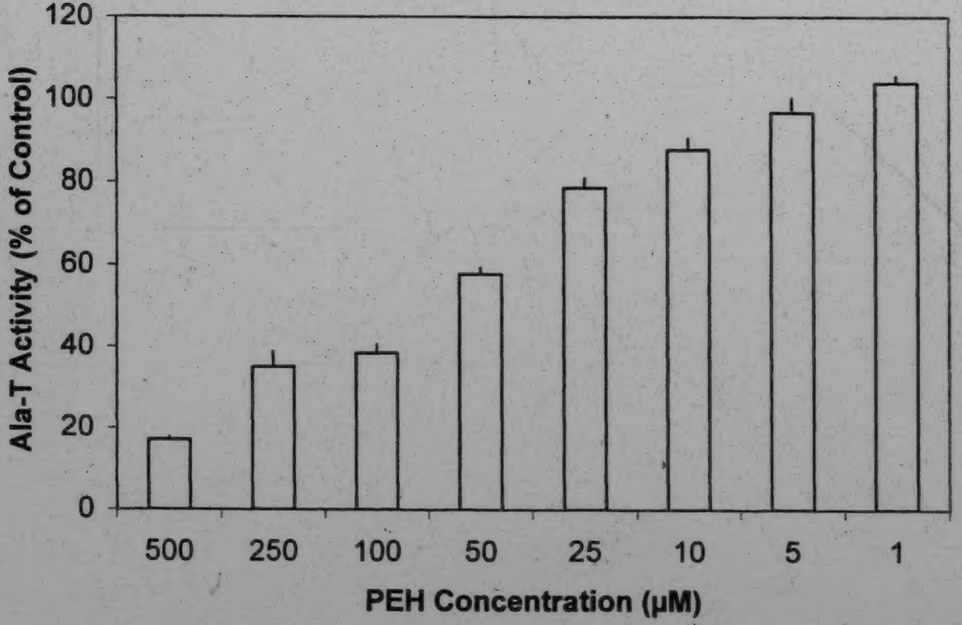


Figure 12. Concentration vs. GABA-T activity (top) and ala-T activity (bottom) in *in vitro* enzyme assays with gerbil whole brain homogenate and PEH (N=5-6).

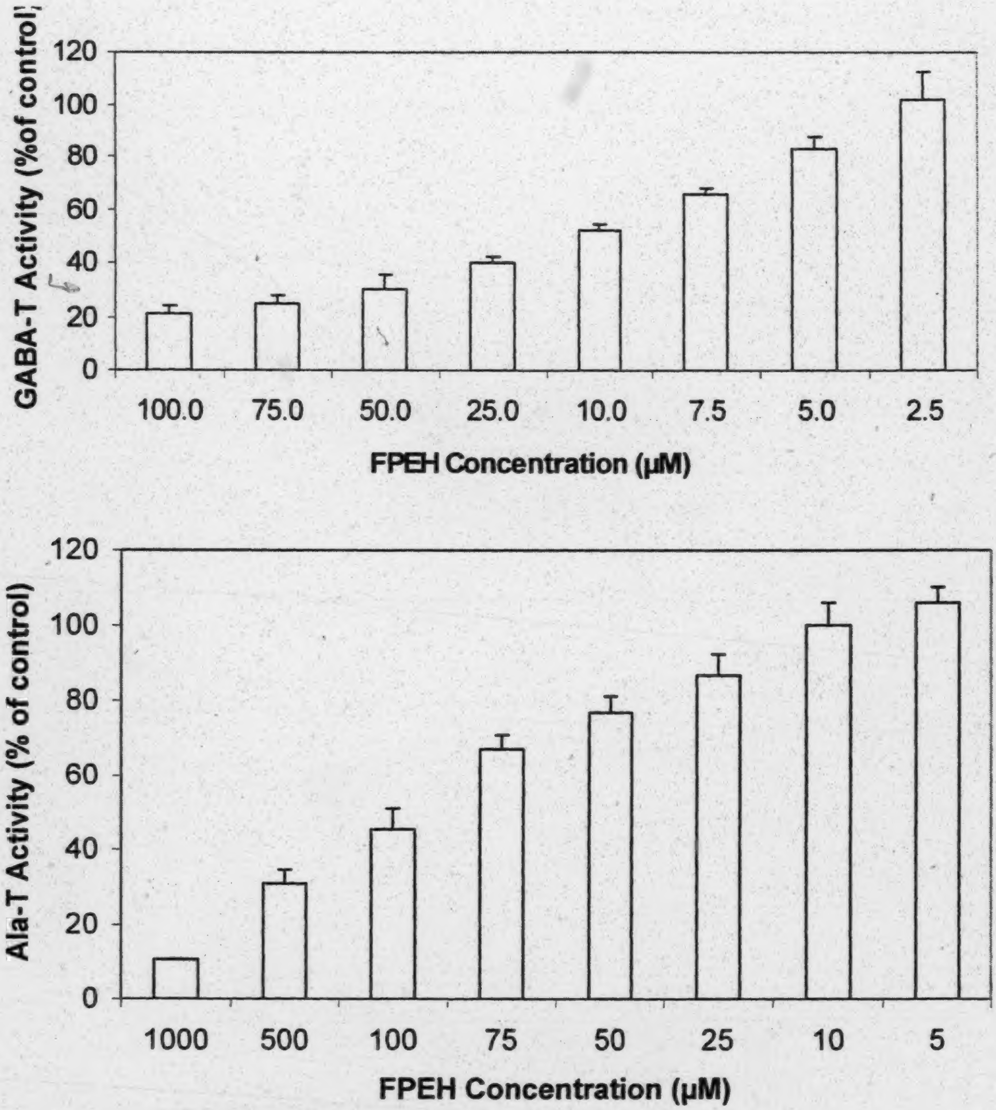


Figure 13. Concentration vs. GABA-T activity (top) and ala-T activity (bottom) in *in vitro* enzyme assays with gerbil whole brain homogenate and FPEH (N = 5-6). Results are expressed as means ± S.E.M.

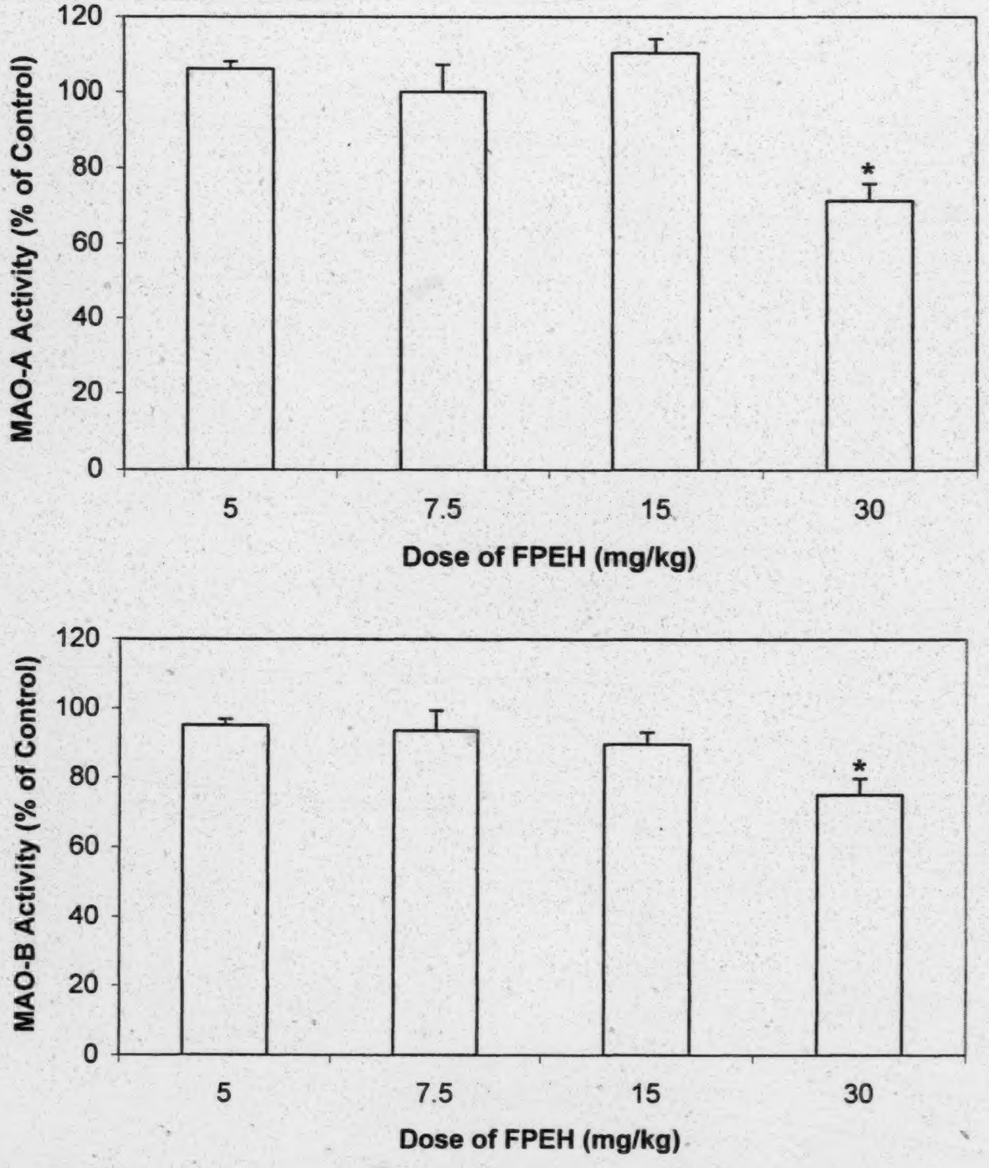


Figure 14. Dose-response study of the effects of FPEH on MAO-A (top) and MAO-B (bottom) activity *ex vivo*. Results are expressed as means ± S.E.M. * $p < 0.05$ compared to control values.

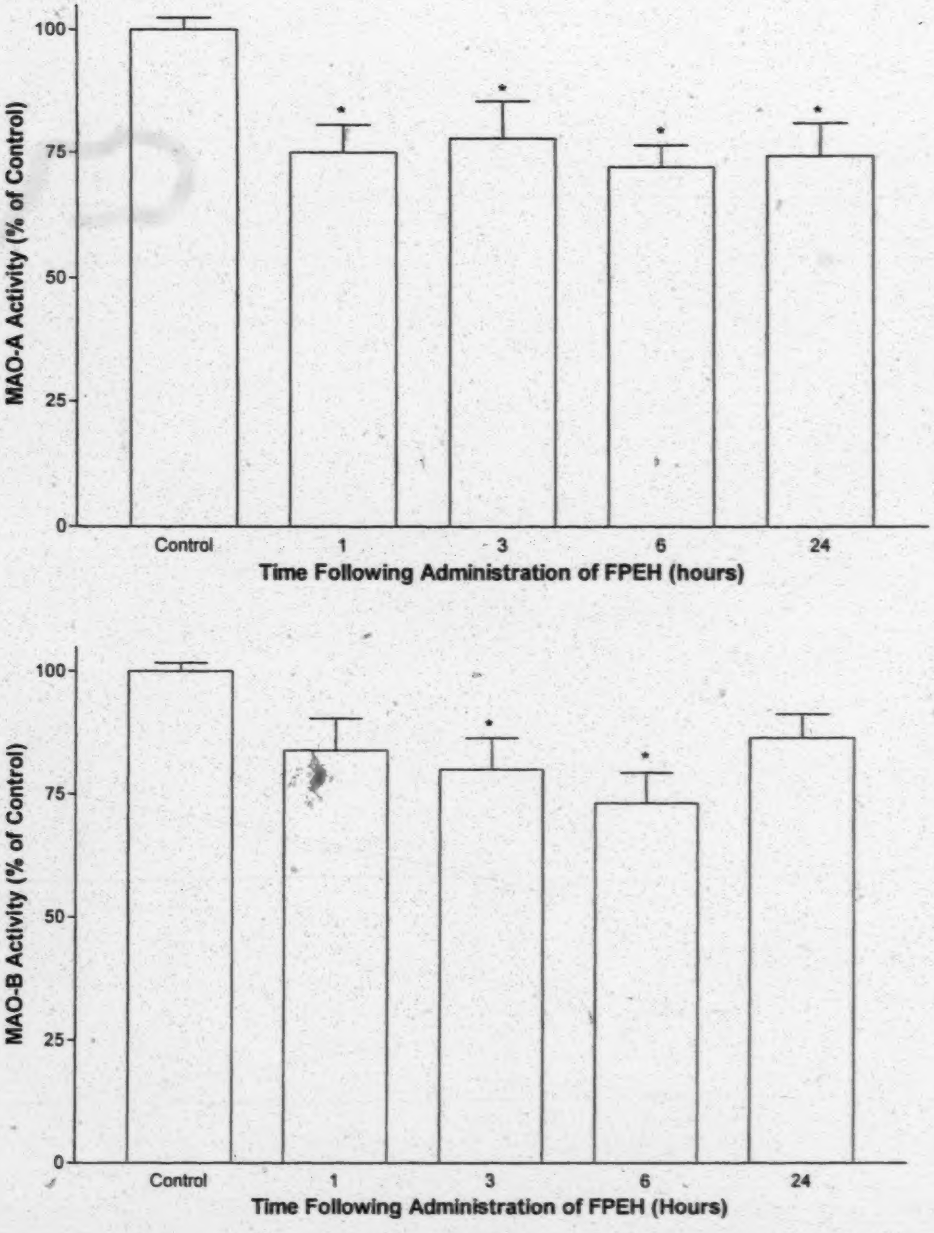


Figure 15. Time-study of effects of FPEH (30 mg/kg i.p.) on MAO-A (top) and MAO-B (bottom) activity 4 hours after drug administration. Results represent means ± S.E.M. (N=6).

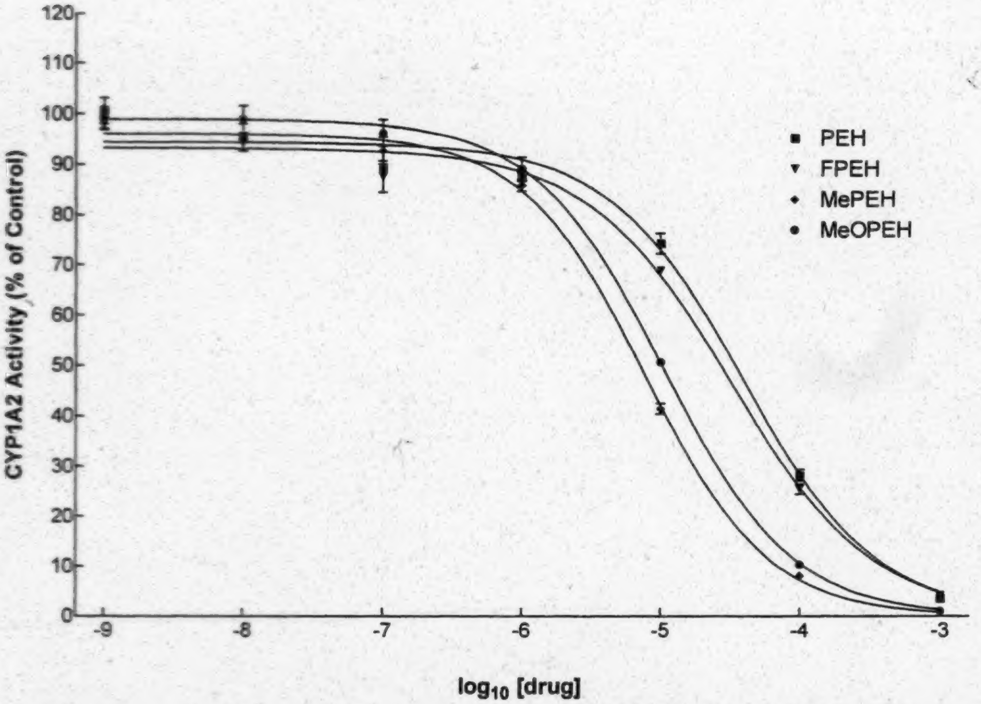


Figure 16. Effects of PEH and PEH analogues on CYP1A2-associated metabolic activity. Results are expressed as means ± S.E.M. (N=3).

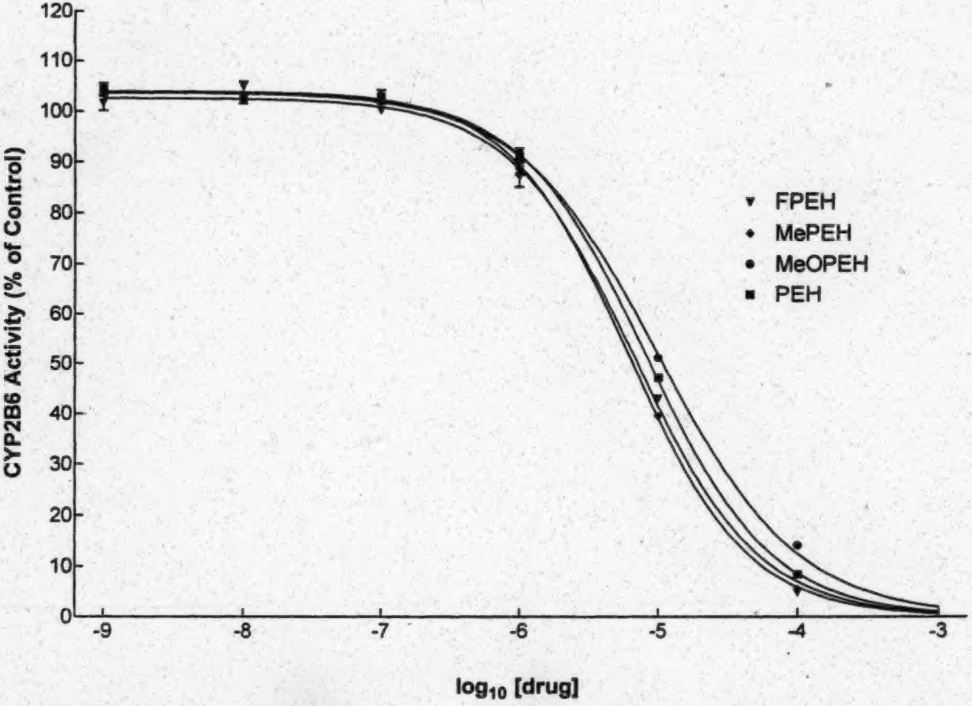


Figure 17. Effects of PEH and PEH analogues on CYP2B6-associated metabolic activity. Results are expressed as means ± S.E.M. (N=3).

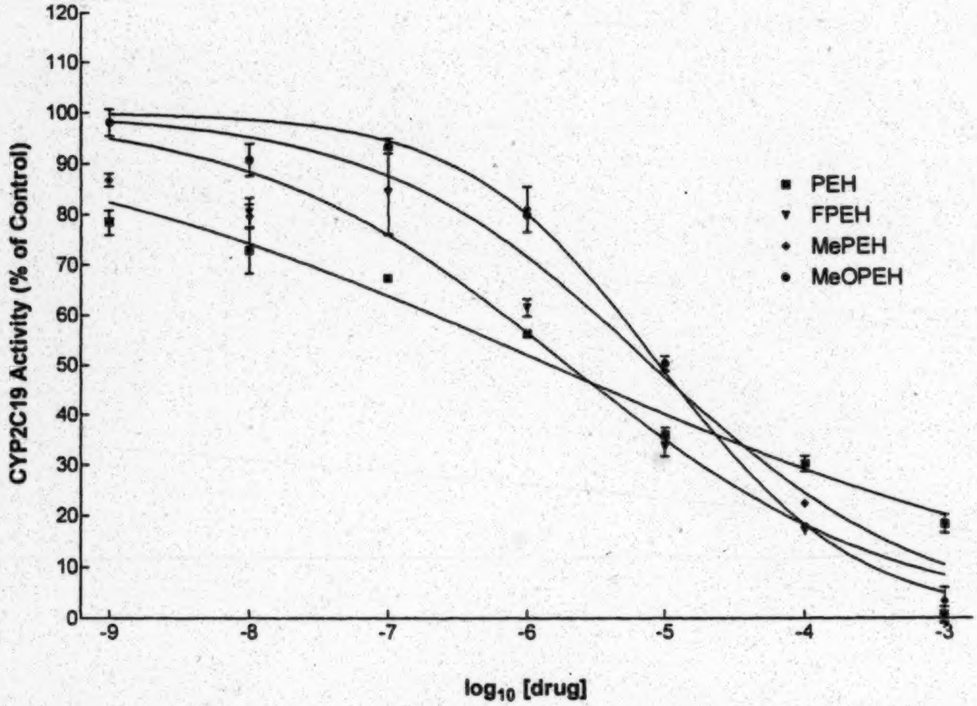


Figure 18. Effects of PEH and PEH analogues on CYP2C19-associated metabolic activity. Results are expressed as means ± S.E.M. (N=3).

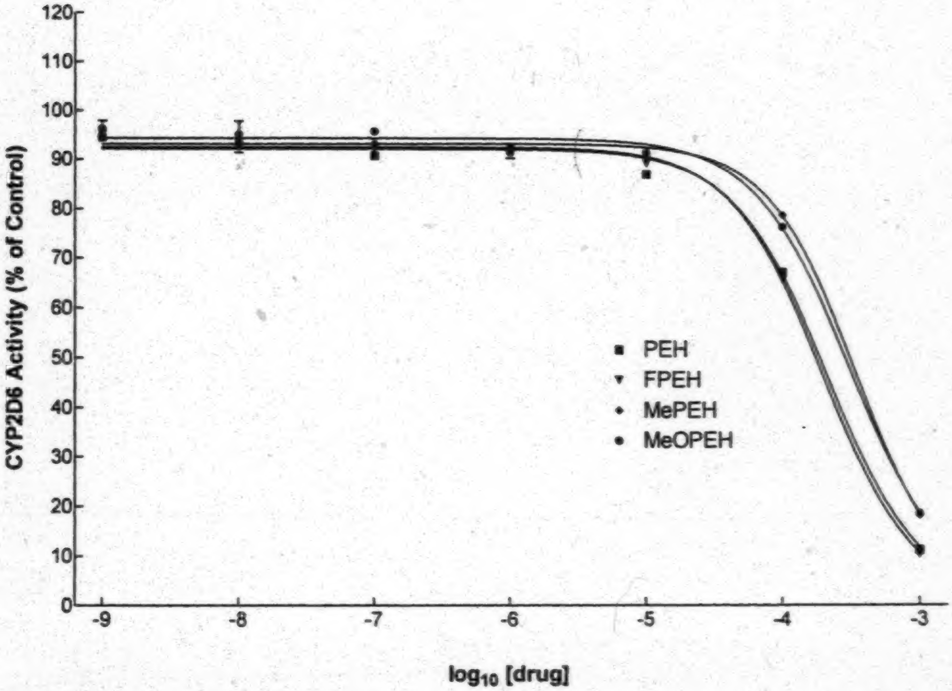


Figure 19. Effects of PEH and PEH analogues on CYP2D6-associated metabolic activity. Results are expressed as means ± S.E.M. (N=3).

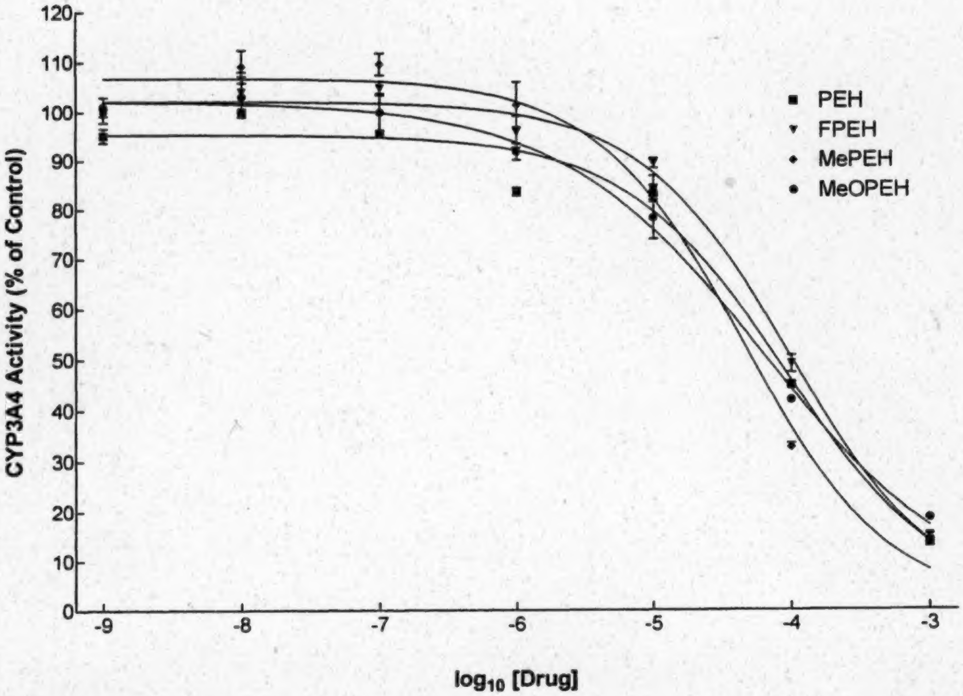


Figure 20. Effects of PEH and PEH analogues on CYP3A4-associated metabolic activity. Results are expressed as means ± S.E.M. (N=3).

CYP Isoform	K_i For Enzyme Inhibition (μM)			
	PEH	FPEH	MePEH	MeOPEH
1A2	16 ± 2	13 ± 2	3.3 ± 0.2	4.4 ± 0.1
2B6	4.0 ± 0.2	3.3 ± 0.1	3.0 ± 0.1	4.9 ± 0.2
2C19	3.9 ± 1	3.5 ± 0.9	10 ± 2	6.5 ± 0.5
2D6	87 ± 5	81 ± 4	142 ± 7	129 ± 6
3A4	72 ± 10	77 ± 9	35 ± 6	48 ± 4

Table 2. K_i values for inhibition of CYP enzyme activity. Results are expressed as means ± SEM (N = 3)

PLZ) and their potential metabolism to biologically active or inactive compounds, including some compounds that may have completely different pharmacological targets. In the present study, several analogues of PEH were examined for their effects on brain GABA-T, ala-T, MAO and CYP isozyme activity.

PEH and FPEH dose-dependently inhibited brain ala-T and GABA-T *ex vivo* (Fig. 10). These effects were presumably responsible for the previously-described effects of these agents on GABA and alanine levels in the brains of drug-treated animals (Paslawski et al., 2001 and Fig. 6). It is of interest that GABA and alanine values increased markedly (see Chapter 3) even when GABA-T and ala-T were inhibited by less than 50%. This is similar to results obtained previously by other workers using similar doses of PLZ in rats (Popov and Matthies, 1969; Baker et al., 1991; Tanay et al., 2001). In dose-response studies, Popov and Matthies (1969) did not find greater than 50% inhibition of GABA-T at any dose of PLZ in rats. PEH and FPEH were also studied *in vitro* with regard to their abilities to inhibit GABA-T and ala-T (Figures 12 and 13), and it was shown that they did so in a dose-dependent manner. This is the first time, to our knowledge, that the effects of these drugs have been studied *in vitro* in gerbil brain; their effects on GABA-T *in vitro* have been investigated in a preliminary manner in rat brain (Sowa et al., 2005). Once an assay for these compounds is developed, it will be of interest to compare the effects of these compounds on the enzymes *ex vivo* and *in vitro* to the actual concentrations of the compounds in brain.

The weak inhibition of MAO-A and MAO-B by FPEH is similar to what was reported by Paslawski et al., (2001) for PEH in rat brain and is marked contrast to the potent, long-lasting inhibition produced by PLZ. The degree of inhibition seen with FPEH is much less than the 65% required to produce any increases in brain levels of biogenic amines in rats (Hampson et al., 1986) or the 80% inhibition required before clinical improvement is observed in humans (Robinson et al., 1980).

Inhibition of CYP enzyme-mediated metabolism and the characterization of these inhibitory actions are important, particularly if the utility of these and related compounds in treating medical conditions is considered. Coadministration of multiple drugs (polypharmacy) may place patients, such as psychiatric or neurologic patients, at high risk for pharmacokinetic drug-drug interactions, which are often mediated by CYP enzymes. The CYP enzymes tested here represent those responsible for the metabolism of the large majority of commercially available drugs. Although the work described here on CYP ezymes does not represent a comprehensive study on enzyme kinetics, based on the K_i values obtained, it appears that PEH and the three analogues are all more potent inhibitors of CYPs 1A2, 2B6 and 2C19 than of 2D6 and 3A4. As mentioned in the introduction, CYPs 1A2, 2B6 and 2C19 are involved in the metabolism of many therapeutic drugs such as antidepressants and benzodiazepines (Bloomer et al., 1992; Caccia, 1998; Greenblatt et al., 1998; Fleishaker, 2000; Herrlin et al., 2003; Eap et al., 2004). Considering the high lifetime risk of major depressive disorder, the prevalence of atherosclerotic disease worldwide and the high degree of comorbidity with these conditions, the potential for antidepressant agents to be co-administered with stroke therapy is great enough to necessitate careful consideration of the interactions of the drugs used in the treatment of each.

Experiments such as those conducted with the CYP screening procedures described here indicate that the drugs will interact with CYP enzymes, but do not indicate if they act as competitive substrates, inhibitors or both. Further studies in which metabolism of PEH and its analogues is investigated in the presence and absence of these CYP enzymes (cDNA-expressed CYP enzymes are commercially available) will be necessary to determine if these drugs are substrates for these enzymes, but at present no methods for analysis of the PEH analogues are available. Based on the results founds in the present study, PEH and its three analogues appear to be more potent inhibitors of CYPs 1A2, 2B6 and 2C19 than of CYPs 2D6 and 3A4, but more comprehensive enzymatic studies at various concentrations will have to be conducted before definitive comparisons can be made. It is anticipated that PEH will be metabolized via CYP2D6-mediated ring hydroxylation at the 4 position of the phenyl ring since many drugs with a phenyl ring and ethyl side chain with an amine group on it (e.g. amphetamines) are metabolized in that fashion. McKenna et al. (1990) also presented evidence for the ring hydroxylation of PLZ. The ring-substituted PEH analogues should be protected against such metabolism, but the fact that they have K_i values for inhibition of CYP2D6 similar to that for PEH suggest that they are inhibitors of this CYP isozyme, even if they may not be substrates.

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Chapter 5

Neuroprotection and Chronic Neurochemical Effects of PEH Analogues in Gerbil Global Ischemia

5.1. Introduction

In cerebral ischemia, an imbalance between excitatory glutamatergic activity and inhibitory GABAergic activity is thought to result in neuronal damage via excitotoxic mechanisms (Schwartz-Bloom and Sah, 2001). Experimentally, two different approaches have been employed towards reducing neuronal injury and death in this context: (A) reducing glutamate receptor activation; and (B) increasing GABAergic activity.

The results of clinical trials of glutamate receptor antagonists in human stroke patients, unfortunately, have been far less hopeful than had been anticipated (Muir and Lees, 2005). The use of glutamate receptor antagonists in humans is limited by neuropsychiatric and peripheral side-effects including confusion, hallucinations and hypertension (Lees, 1997; Schwartz-Bloom and Sah, 2001). An alternative approach towards normalizing the balance between glutamatergic and GABAergic activity in the setting of cerebral ischemia is to augment the activity of GABAergic systems (Shuaib and Kanthan, 1997; Shuaib et al., 1997).

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the human central nervous system (Cooper et al., 2003). The activity of GABAergic signaling pathways can be increased by (1) increasing GABA release, (2) using positive regulators of GABA receptors, (3) adding $GABA_A$ receptor agonists, (4) reducing the reuptake of GABA by inhibiting GABA transport proteins, and (5) reducing the catabolism of GABA.

Clomethiazole is a GABA-mimetic agent that both potentiates the activity of endogenous GABA at $GABA_A$ receptors and can also directly activate $GABA_A$ receptor-mediated chloride flux across neuronal membranes, although the mechanism by which the latter effect occurs has not been determined (Sydserff et al., 2000; Lyden et al., 2002). The promise shown by this drug in the majority of pre-clinical neuroprotection studies (Shuaib et al., 1995; Cross et al., 1991; Marshall et al., 1999; Sydserff et al., 2000) led to two well-designed, randomized, controlled studies of the effectiveness of clomethiazole in improving clinical outcome in human stroke patients. The **cl**omethiazole **a**cute **s**troke **s**tudy (CLASS)(Wahlgren et al., 1999; Lyden et al., 2001, 2002) returned negative results with respect to the efficacy of clomethiazole at improving patient recovery following middle cerebral arterial (MCA) stroke. This was disappointing given the potential that clomethiazole displayed in pre-clinical studies. One proposed explanation for the failure of clomethiazole in human trials is difficulty in translating animal doses into human doses (Curry, 2003).

Whereas clomethiazole appears to potentiate GABAergic activity via direct interaction with the $GABA_A$ receptor (Cross et al., 1989; Green et al., 2000), other drugs such as PLZ and PEH reduce the metabolic breakdown of endogenous GABA (McManus et al., 1992; Paslawski et al., 2001), thereby increasing brain GABA levels (Parent et al., 2002). The goal of this approach is to prolong the duration of time that endogenous GABA remains present and capable of counteracting, via activation of $GABA_A$ receptor-associated inhibitory chloride fluxes, glutamate receptor-associated excitation. While PLZ and PEH are potentially susceptible to metabolic modification, such as hydroxylation at the *para* position of their phenyl rings (McKenna et al., 1990), the PEH analogues FPEH, MePEH and MeOPEH are protected by substitution at that position and may even be expected to persist in the body for a longer duration following administration as a result of this.

FPEH, MePEH and MeOPEH were examined with respect to their potential as neuroprotective agents in two separate systems: (1) gerbil transient forebrain ischemia; and (2) a rat model of DSP-4 neurotoxicity (Chapter 6). In this chapter, the neuroprotective actions of the analogues in the gerbil global ischemia model are described, as well as their chronic effects on amino acids in gerbil frontal cortex. Data from the National Institute of Neurological Disorders and Stroke clinical trial of recombinant tissue plasminogen activator (rt-PA; activates plasminogen to plasmin, facilitating the breakdown of fibrin clots) in the treatment of acute stroke showed that stroke patients treated with rt-PA within 3 hours of the onset of stroke symptoms displayed significantly improved outcome at 3 months post-stroke (The National Institute of Neurological Disorders and Stroke, 1995). Accordingly, the accepted time window for acute treatment of ischemic stroke is now generally viewed as the 3-6 hour period following the onset of stroke symptoms. All PEH analogues investigated for neuroprotective effects in the gerbil global ischemia model were thus administered at 3 hours post-reperfusion.

5.2. Methods

5.2.1 Surgical Procedure and Drug Treatment (Transient Forebrain Ischemia)

All surgical subjects were anesthetized to the level of surgical anesthesia using a mixture of oxygen (80%) and nitrous oxide (20%), into which halothane was introduced at varying concentrations depending on whether the intent was induction of anesthesia (3-5%) or maintenance of animals in the surgical plane (1.5-2%). Once the level of surgical anesthesia was reached, as determined using the toe-pinch reflex, the ventral surface of the neck was shaved and cleaned. A ventral midline neck incision was then made and blunt dissection was used to expose both common carotid arteries (CCAs). Occlusion of arteries was achieved using external mechanical pressure applied by microaneurysm clips. Flow was re-established (i.e. reperfusion occurred) five minutes following clipping of the arteries by removal of the aneurysm clips and sutures. Surgical wounds were stitched closed and animals were observed as they recovered in their cages, with free access to food and water. In sham-operated animals the CCAs were isolated but occlusion of the arteries was not performed. Hyperactivity for 1-2 days following the operation was typically observed in gerbils that had been subject to transient forebrain ischemia. Animals were administered drug or vehicle (corn oil) intraperitoneally at 3 hours post-reperfusion and then once daily for 7 days. On the 8th day post-reperfusion, i.e. 24 hours after the last injection, the animals were decapitated and their brains were isolated and frozen for use in several experimental procedures, as is described below.

5.2.2 Preservation of Frontal Cortex for HPLC Analysis

Since occlusion of the common carotid arteries results in ischemia in the entire forebrain, the frontal cortex of the gerbils used in these experiments was chosen for amino acid analysis in order to observe the effects of ischemia and drug treatment on amino acid levels in brain tissue that has endured transient ischemia. Hippocampus could not be used in this assay since it was to be used in the H&E and GFAP staining procedures. A blade was used to coronally slice 3-4 mm of frontal cortex from the brain of each gerbil used in the neurorescue experiments. That portion of frontal cortex was saved for later HPLC analysis of amino acid levels. Isolation of frontal cortex was performed immediately prior to mounting of brains for sectioning and subsequent hematoxylin & eosin (H & E) staining or staining for glial fibrillary acidic protein (GFAP).

5.2.3 Staining of Brain Sections

Determination of neuronal survival can be achieved, using light microscopy, by manual counting of healthy-appearing neurons in brain sections from experimental animals. Staining of the sections with dyes such as hematoxylin and eosin facilitates visualization, identification and counting of cells of interest. Brains obtained from animals used in the neurorescue experiments were sliced into 20μm sections using a cyrostat (Leica CM 1850 purchased from Leica Microsystems Nussloch, GmbH, Germany). Sections were transferred to microscope slides and then stained using either H & E for neuronal counts or immunohistochemically for GFAP (for qualitative examination of reactive astrocytosis, an indirect measure of neurotoxic insult). The procedure for H&E

staining involved sequential bathing of microscope slides with the following: xylene, 1% zinc chloride, 90% ethanol, double-distilled water (ddH_2O), hematoxylin, ddH_2O, $HCl_{(aq)}$, ddH_2O, 1% ammonium hydroxide$_{(aq)}$, ddH_2O, eosin, ddH_2O, 100% ethanol, and xylene. Following these steps, cover glasses (Fisher*finest* Premium Cover glass, Fisher Scientific Co., USA) were then placed on slides using Permount^{TM} and the slides were allowed to dry for at least 1 day prior to use in microscopy. Determination of neuronal survival involved microscopic examination and counting of healthy-appearing neurons in hippocampal region CA1 and expression of cell counts relative to counts obtained in healthy control animals. When performing neuronal counts the 20X objective lens was used in combination with the 10X ocular lens of the microscope, yielding a total magnification of 200X. For each hippocampus, healthy-appearing pyramidal cells in three adjacent visual fields within the CA1 region were counted. As mentioned earlier, H & E staining allows for visualization and enumeration of cells of interest via light microscopy. It aids in visualizing cells but, unlike staining for GFAP (discussed later), does not preferentially stain cells of one particular type. Hematoxylin is a basic dye that stains nuclei, ribosomes and rough endoplasmic reticulum blue in colour due to their high DNA or RNA content. Eosin, an acidic dye, stains basic components, such as cytoplasmic proteins, pink in colour (Young and Heath, 2000).

Fixation of microscope slide-mounted tissue prior to GFAP staining was performed on slices adjacent to those used in H & E staining, using formalin. Tissue was then subjected to a chemical "defat" using 70%, 90% and 100%

ethanol and xylenes prior to GFAP staining. The stepwise procedure employed for staining of GFAP was as follows: 1% H_2O_2, rinse, proteinase K, rinse, universal blocker + 0.1% triton X-100, primary antibody (rabbit anti-cow GFAP; 1/750 dilution) rinse, secondary antibody (goat anti-rabbit IgG; 1/200 dilution), rinse, avidin-biotin complex (1/200 dilution), rinse, diaminobenzidine, rinse, dehydration with ethanol followed by xylenes. Following the staining procedure, cover glasses were placed on the slides, using Permount™ and slides were permitted to dry for a minimum of 24 hours prior to their use in microscopy. Each slide was examined by light microscopy and assigned a score based on its relative density of staining for GFAP (arbitrary scale). Once all slides had been examined, scores were collected for each of the treatments (SHAM+VEH, SHAM+Drug, ISCH+VEH and ISCH+Drug). In contrast to the non-cell-type-specific staining achieved with H & E (described earlier), staining for GFAP allows identification and examination of a specific cell type, namely the astrocyte. GFAP is a glial structural protein that connects cellular structures to each other and to plasma membrane proteins. Astrocytic activation in response to brain trauma or toxic insult is associated with hypertrophy of astrocytic processes (astrocytic gliosis), which involves increased production of GFAP and other intermediate filaments (Young and Heath, 2000; Pekny and Pekna, 2004). Staining of astrocytes using GFAP combined with light microscopy facilitates visualization of the thickening of astrocytic processes that is a component of reactive gliosis (Pekny and Pekna, 2004).

5.3. Results

5.3.1 Neuroprotective Effects of PEH Analogues in Transient Forebrain Ischemia

FPEH administration at 3 hours post-reperfusion and then once daily for 7 days post-reperfusion was associated with a doubling of hippocampal CA1 cell survival relative to that seen in animals treated with vehicle (Fig. 21a). Hippocampal CA1 neuronal survival was 36% in animals treated with FPEH, compared to only 18% in animals given vehicle alone. In animals treated with MePEH there was a trend towards increased neuronal survival; however, the difference in CA1 cell survival between vehicle and MePEH-treated animals was not statistically significant (Fig. 21b). A neuroprotection study with MeOPEH was conducted. While the histochemistry portions of that study were completed by Mr. Sean Galante of the Department of Pharmacology, and the resultant data therefore not shown (although no neuroprotective effect was observed), I co-directed his project and co-performed the neurochemistry study with MeOPEH, the data for which are included in the following section.

5.3.2 Effects of PEH Analogues on Amino Acid Levels in Ischemic Animals

No significant differences in alanine and glutamine levels were observed in the ISCH+VEH and ISCH+drug animals for any of the drugs (ANOVA followed by Neuman-Keuls test, $p > 0.05$). These findings were in contrast to those with GABA (increased in all three ISCH+drug groups relative to ISCH+VEH) and glutamate (decreased in the ISCH+FPEH group versus the ISCH+VEH group) (Figs. 22-27).

In ISCH+FPEH animals, brain GABA levels were elevated relative to ISCH+VEH animals (but not to the same extent as seen in SHAM+FPEH animals) (Fig. 22) while brain glutamate levels in ISCH+FPEH animals were significantly lower than those seen in ISCH+VEH animals (Fig. 23).

Increased levels of GABA were also seen in frontal cortex from ISCH+MePEH and ISCH+MeOPEH animals relative to ISCH+VEH animals, and the extent of the increases was not significantly different from that seen in the essentially uninjured SHAM+MePEH animals (Figs. 24 and 26). While, as mentioned above, ISCH+FPEH gerbils had reduced brain levels of glutamate relative to the ISCH+VEH animals, brain levels of glutamate were not affected by MePEH or MeOPEH treatment of ischemic animals (Figs. 25 and 27, respectively).

5.3.3 Effects of PEH Analogues on Reactive Gliosis in the Setting of Cerebral Ischemia

Staining for GFAP in sections prepared from the brains of ±ISCH ±PEH analogue gerbils revealed a lesser degree of astrocytosis/astrocytic hypertrophy in FPEH-treated and MePEH-treated (Fig. 28) animals when compared to vehicle-treated ischemic animals.

5.4. Discussion

The data reported here reveal differences in the chronic neurochemical effects of FPEH, MePEH and MeOPEH on GABA and glutamate in frontal cortex following administration in the setting of cerebral ischemia. These differences may account, in part, for the differences in the neuroprotective efficacy of the drugs in the gerbil forebrain ischemia model. It should be noted that, while the data are not shown here since the neuronal cell count study with MeOPEH was performed by another investigator, MeOPEH was found to be inactive as a neuroprotective agent in the gerbil global ischemia model; difficulty in obtaining additional amounts of the MeOPEH after initial experiments resulted in its exclusion from some of the studies in other chapters within this thesis.

Interestingly, while increases in brain GABA levels were seen following repeated daily injections of the analogues to SHAM and ischemic animals, differences were seen between the drugs with respect to their effects on brain glutamate levels. Just as FPEH decreased brain glutamate levels acutely in healthy gerbils (see Chapter 3), that drug appears to maintain its glutamate-lowering effect with repeated daily administration for seven days. Although in the acute study 30 mg/kg FPEH did not produce significantly lower levels of glutamate relative to controls, daily administration of that dose of FPEH to ischemic animals for 7 days (Fig. 23) was associated with a reduction in brain glutamate levels. The reduction in brain glutamate content associated with FPEH administration to ischemic animals may contribute to the neuroprotective effect of FPEH.

Glutamate levels in the ISCH+MePEH animals relative to the ISCH+VEH animals showed a trend towards a decrease, but this was not significant. Studies on an increased dose of the drug may be warranted, but sedation is a problem with all of the analogues at doses above 30 mg/kg.

It is reasonable that inhibition of GABA-T by the PEH analogues would result in a decrease or no change in brain glutamate levels, as conversion of GABA to succinic semialdehyde by GABA-T is coupled with the conversion of alpha-ketoglutarate to glutamate (Cooper et al. 2003); inhibition of this activity removes one source for the production of glutamate. The lack of a simple, direct method for predicting changes in brain glutamate following changes in brain levels of GABA and other neurotransmitters stems from the fact that there are several routes by which glutamate can be produced, including several transamination reactions (Holt et al., 2004).

Reactive astrocytosis/gliosis has been described following ischemia in experimental animals, is indicative of a toxic insult and neuronal injury, and varies in severity in a manner that reflects the severity of the insult (Petito and Halaby, 1993; Schroeter et al., 1995; Kumar et al., 1996; Kunkler and Kraig, 1997; Bernaudin et al., 1998; Lin et al., 1998). Neuroprotective interventions such as hypothermia (Kumar et al., 1996) have been shown to decrease ischemia-induced astrocytosis, as indicated by relatively reduced GFAP staining intensity of astrocytes as well as relatively reduced thickness of astrocytic processes. In the present study, treatment with FPEH (Fig. 28) resulted in reductions in astrocyte hypertrophy, and the effect was consistent with the reduction in neuronal death seen with FPEH treatment.

In addition to demonstrating that FPEH is neuroprotective when administered 3 hours post-reperfusion in the gerbil model of transient forebrain ischemia (Fig. 21) and reduces the extent of astrocyte reactive hypertrophy following an ischemic insult (Fig. 28), the data presented here suggest the importance of investigating the effects of GABAergic drugs, or at least inhibitors of GABA-T, on the balance between brain GABA and glutamate levels in both control and ischemic animals. Administration of the dose of drug producing the *greatest effect* on indices of GABA receptor activation (e.g. chloride currents, induction of hypothermia) or indices of GABAergic augmentation (eg. inhibition of GABA-T) may prove to be less efficacious than administration of the drug dose producing the *most favourable combination of changes* in several indices, such as the concentrations of glutamate and GABA. This is not to say that other neurochemicals should not be considered, but only to draw conclusions based on the data obtained in these studies. It is quite possible that a sub-maximal elevation of brain GABA levels combined with significant reduction of brain glutamate levels will be at least as efficacious in currently used neurorescue paradigms as drug concentrations producing maximal increases in brain GABA levels coupled with lesser-to-no reduction in brain glutamate levels.

Previous studies under the same conditions (i.e. administration of the drug 3 hours after reperfusion and daily administration for 7 days) with PLZ and PEH showed that those two drugs also caused increased neuronal survival (Todd et al., 1999; Tanay et al., 2002). However those studies were not accompanied by analysis of levels of amino acids in brain. In fact, to my knowledge, this is the first study to examine the effects of ischemia and drug treatment on neuronal survival, reactive gliosis and levels of GABA, glutamate, alanine and glutamine, all in the same animals.

Although FPEH produced a decrease in glutamine levels at 4 hours in the acute neurochemical study (Fig. 7), a decrease in glutamine levels was not noted in the chronic study. In the chronic study, measurements were made 24 hours after the final FPEH administration, by which time glutamine levels had returned to control levels in the acute study. It is not clear at this time why alanine levels, in contrast to GABA levels, did not increase in a pattern similar to those observed in the acute study, but this finding indicates an interesting difference in the chronic effects on GABA and alanine with the PEH analogues.

The lack of elevation of brain alanine could be desirable in the setting of cerebral ischemia since (1) alanine inhibits glutamine synthetase, reducing conversion of glutamate to glutamine with concomitant removal of toxic ammonia; (2) ala-T is an important enzyme in the catabolism of glutamate, and increased glutamate levels in the absence of a change in alanine levels would likely result in an increase in the catabolism of glutamate; (3) alanine positively modulates NMDA receptor activity, although to a weaker extent than does glycine (Chizhmakov et al., 1989). The reduction in brain glutamate would also be desirable since it would hopefully counteract, at least to some degree, the increase in brain glutamatergic activity (and excitotoxicity) seen in the setting of cerebral ischemia.

At the present time it has not been determined whether FPEH simply delays neuronal death or provides long-lasting neurorescue. Tiagabine, a GABA transport uptake inhibitor, and the $GABA_A$ receptor partial agonist imidazenil are neuroprotective at 7 days post-ischemia in rats, but less so at 28 or 35 days post-ischemia (Schwartz-Bloom et al., 1998, 2000). On the other hand, the neuroprotective activity of diazepam in this model has been demonstrated to be robust and long-lasting (Schwartz-Bloom et al., 1998, 2000). In light of such findings it would be of benefit for future studies to examine the long-term effects of potentially neuroprotective compounds on neuronal survival. Additionally, Wang (2003) reported that, *in vitro*, 4 minutes of reduced perfusion results in altered function of hippocampal GABAergic interneurons and a resultant increase in the activity of pyramidal neurons. Accordingly, it may also be recommended that future studies examine not only neuronal survival, but also the functioning of those cells and the neurological performance of the animals.

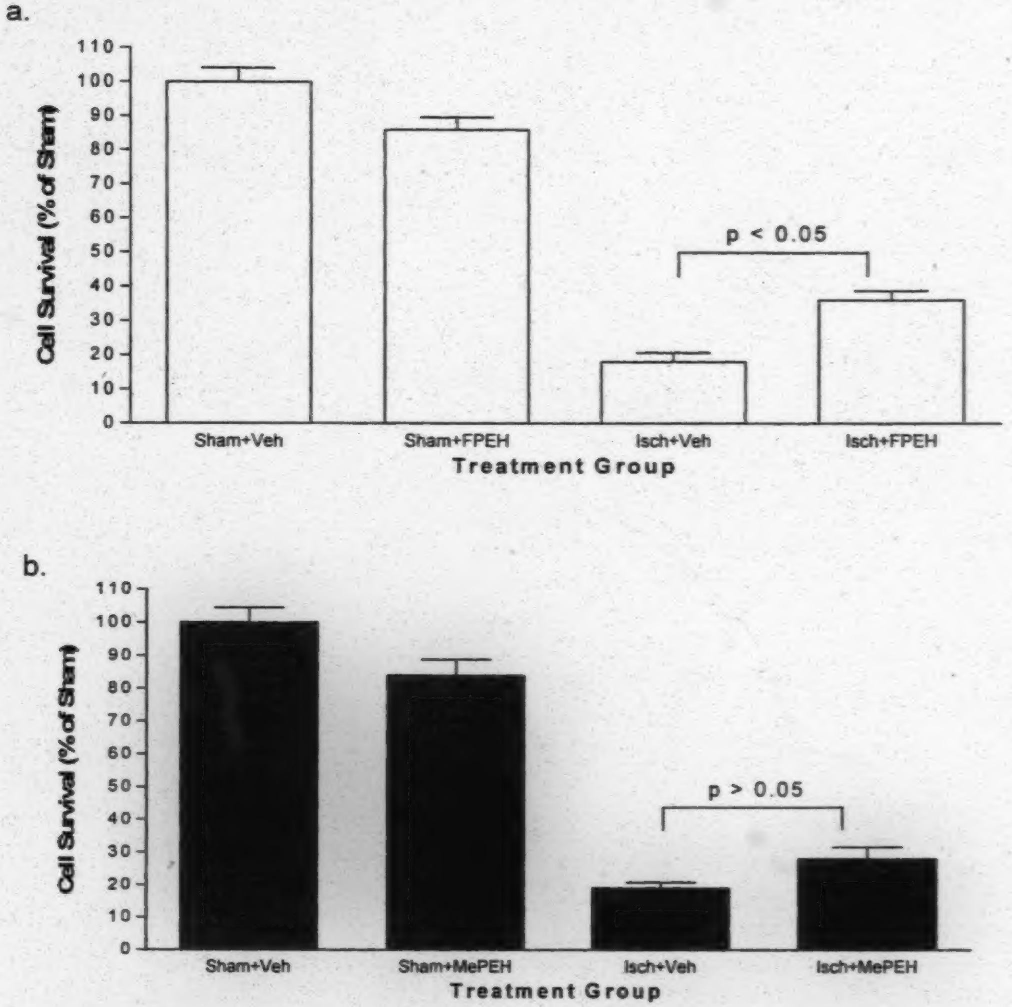


Figure 21. Effects of 30 mg/kg i.p. FPEH (a) and MePEH (b) on the survival of hippocampal CA1 pyramidal neurons in the gerbil transient forebrain ischemia model. Drugs were administered at 3 hours post-ischemia and then once daily for 7 days. Results are expressed as means ± SEM (N=6 animals per group). $p < 0.05$ for all possible comparisons of groups unless otherwise noted.

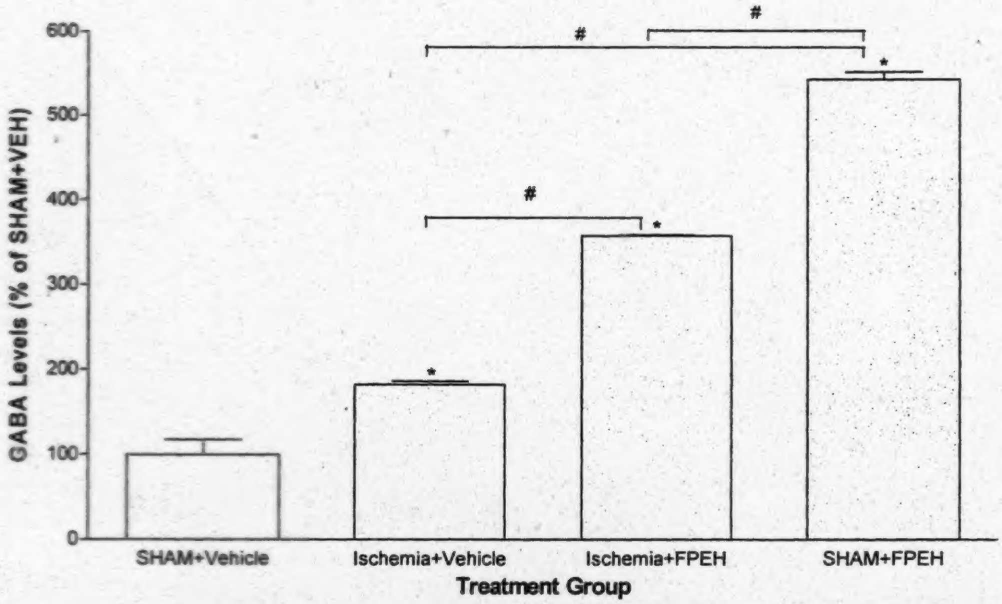


Figure 22. GABA levels in frontal cortices of gerbils (with and without ischemia) after treatment with FPEH or vehicle. Drug/vehicle administration was initiated at 3 hours post-ischemia/SHAM operation and continued for 1 week. Values are expressed as mean ± SEM. N = 5-6 animals per group. * indicates $p < 0.05$ vs SHAM+VEH. # indicates $p < 0.05$ for indicated comparison. VEH = vehicle.

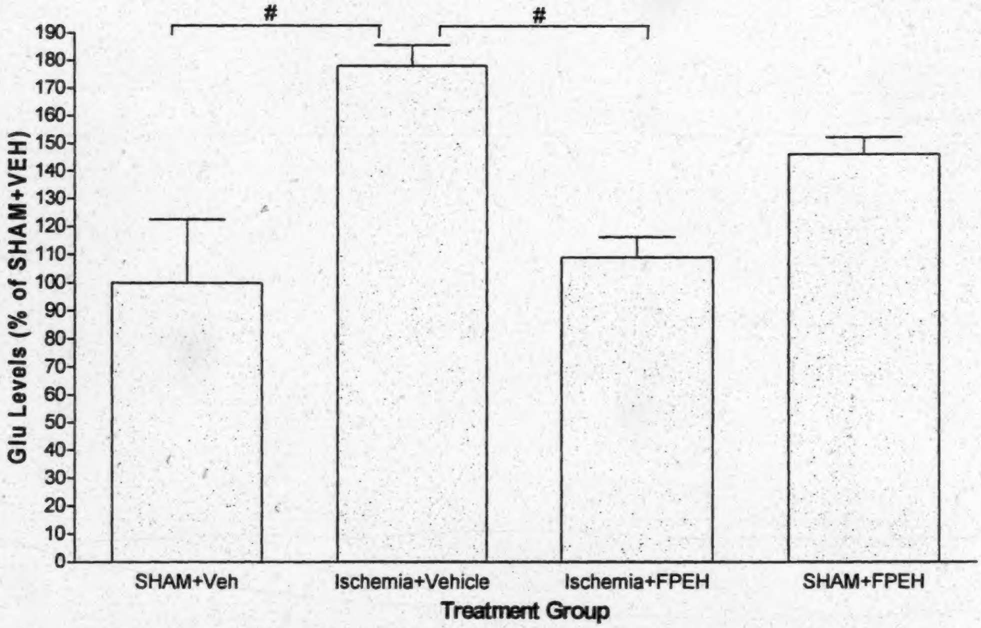


Figure 23. Glutamate levels in frontal cortices of gerbils (with and without ischemia) after treatment with FPEH or vehicle. Drug/vehicle administration was initiated at 3 hours post-ischemia/SHAM operation and continued for 1 week. Values are expressed as mean ± SEM. N = 5-6 animals per group. # indicates $p < 0.05$ for indicated comparison. VEH = vehicle.

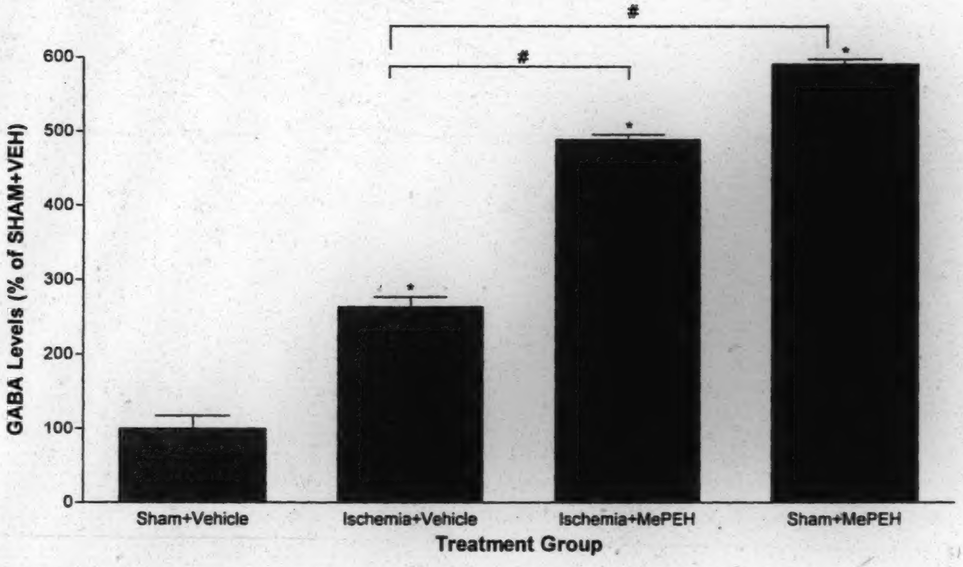


Figure 24. GABA levels in frontal cortices of gerbils (with and without ischemia) after treatment with MePEH or vehicle. Drug/vehicle administration was initiated at 3 hours post-ischemia/SHAM operation and continued for 1 week. Values are expressed as mean ± SEM. N = 5-6 animals per group. * indicates $p < 0.05$ vs SHAM+VEH. # indicates $p < 0.05$ for indicated comparison. VEH = vehicle.

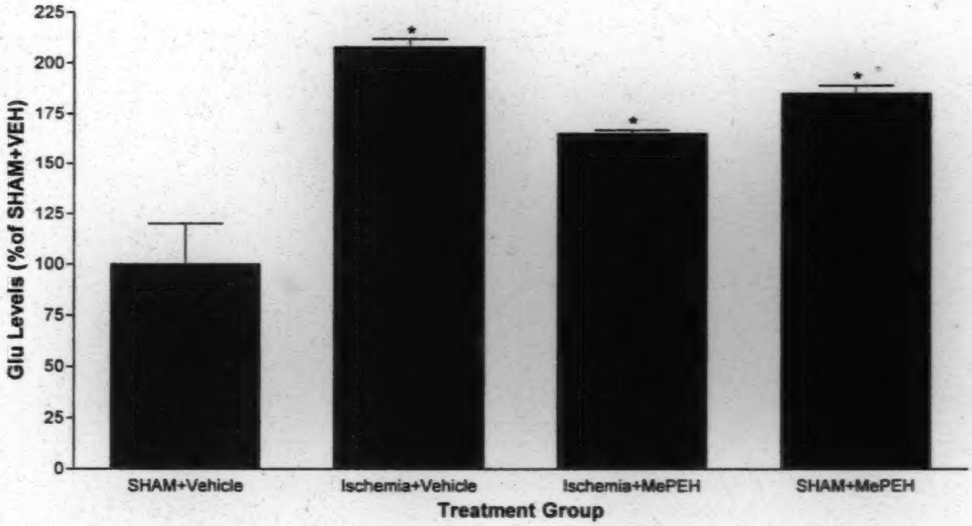


Figure 25. Glutamate levels in frontal cortices of gerbils (with and without ischemia) after treatment with MePEH or vehicle. Drug/vehicle administration was initiated at 3 hours post-ischemia/SHAM operation and continued for 1 week. Values are expressed as mean ± SEM. N = 5-6 animals per group. * indicates $p < 0.05$ vs SHAM+VEH. VEH = vehicle.

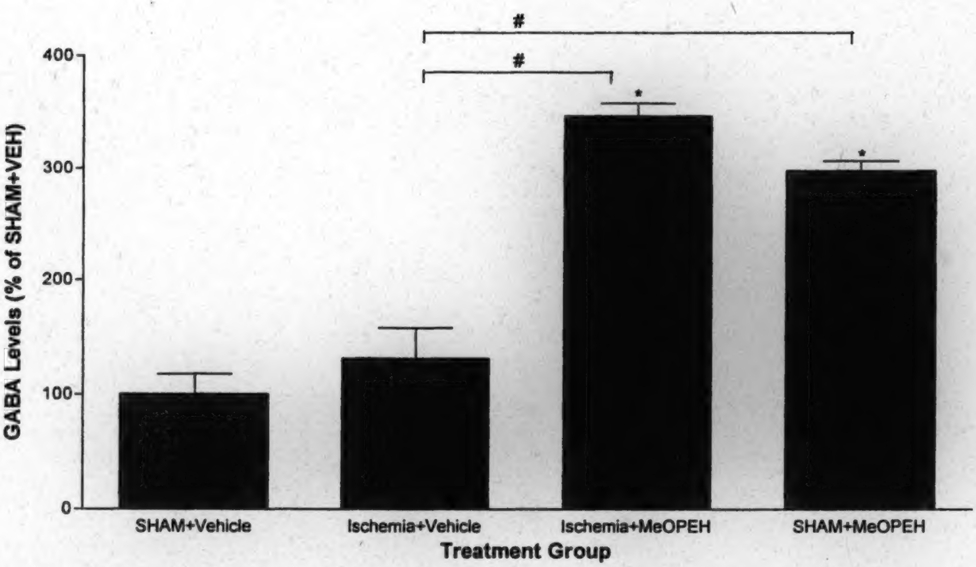


Figure 26. GABA levels in frontal cortices of gerbils (with and without ischemia) after treatment with MeOPEH or vehicle. Drug/vehicle administration was initiated at 3 hours post-ischemia/SHAM operation and continued for 1 week. Values are expressed as mean ± SEM. N = 5-6 animals per group. * indicates $p < 0.05$ vs SHAM+VEH. # indicates $p < 0.05$ for indicated comparison. VEH = vehicle.

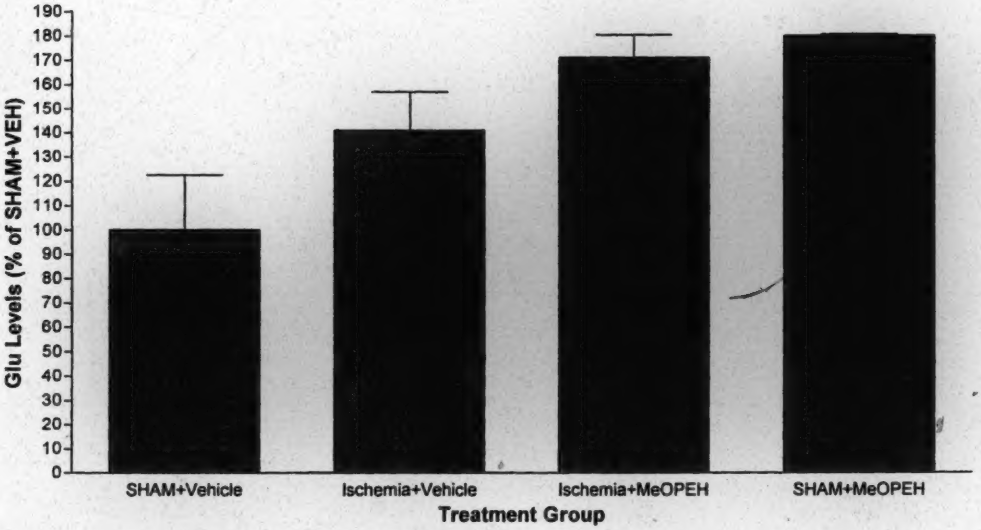
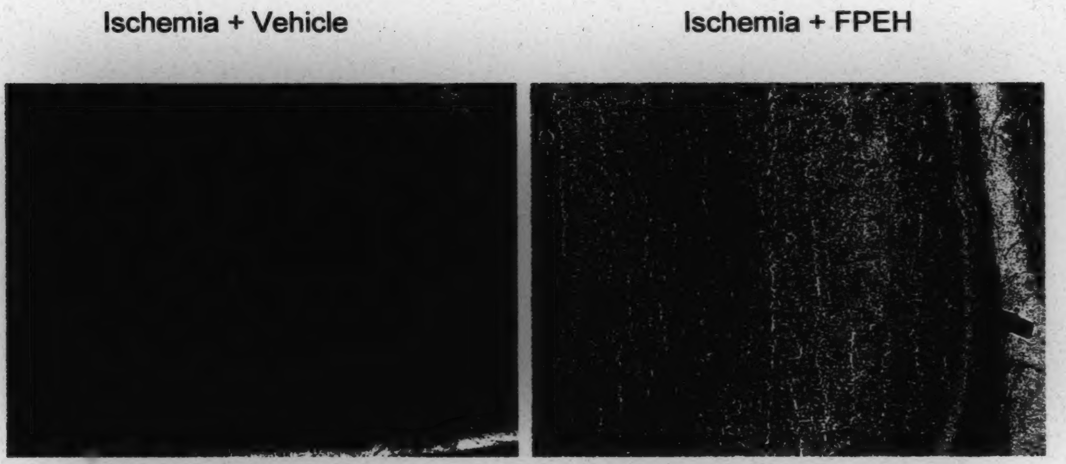


Figure 27. Glutamate levels in frontal cortices of gerbils (with and without ischemia) after treatment with MeOPEH or vehicle. Drug/vehicle administration was initiated at 3 hours post-ischemia/SHAM operation and continued for 1 week. Values are expressed as mean ± SEM. N = 5-6 animals per group. * indicates $p < 0.05$ vs. SHAM+VEH. VEH = vehicle.



Ischemia + Vehicle

Ischemia + FPEH

Figure 28. GFAP-stained sections from Ischemia+Vehicle- (left) and Ischemia+FPEH-treated (right) gerbils. Intensity of brown staining is reflective of the amount of reactive hypertrophy of astrocytes and/or the number of astrocyctes. 100X total magnification.

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Chapter 6

Neuroprotection: DSP-4 Toxicity

6.1. Introduction

Ability to prevent the depletion of hippocampal noradrenaline in rats or mice by *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzyl amine (DSP-4) has been used extensively as a screen for potential neuroprotective drugs. Following uptake into noradrenergic nerve terminals, DSP-4 forms covalent bonds with protein sulfhydryl groups, leading to the eventual death of noradrenergic neurons and depletion of brain noradrenaline (Magyar and Szende, 2004). The essential mechanism(s) involved in the neurotoxic effect of DSP-4 have not yet been determined. The two dominant views in the debate regarding the essential mechanism for preventing DSP-4 toxicity are inhibition of noradrenaline (NA) reuptake and inhibition of MAO-B activity. Studies in which drugs exhibiting various combinations of these two activities have been used in attempting to decrease or prevent DSP-4-associated NA depletion have yielded conflicting data such that there is currently no consensus or even dominating view as to which (if either) of the two mechanisms is key in preventing DSP-4-associated neurotoxicity.

Several reports suggest that NA reuptake is the key to DSP-4-associated neurotoxicity and that inhibition of the uptake of DSP-4 into the terminals of noradrenergic neurons is the key to preventing DSP-4-associated NA depletion. Desipramine, a tricyclic antidepressant drug which inhibits NA reuptake, protects against DSP-4 neurotoxicity (Ross, 1976; Jonsson et al., 1982; Berry et al., 1994), although it as well as *l*-deprenyl were shown to be less effective in this regard than some specific inhibitors of MAO-B, in the hands of Yu and colleagues (1994). *l*-Deprenyl, commonly known for its MAO-B-inhibitory activity, also inhibits NA reuptake and prevents NA depletion in mouse hippocampus when administered one hour prior to DSP-4 administration. Magyar et al. (1998) proposed that it is actually methylamphetamine, a metabolite of *l*-deprenyl, that is responsible for this effect (Magyar et al., 1998). This finding followed experiments by the same group showing that non-selective inhibition of microsomal oxidation using SKF-525A (an inhibitor of the metabolism of deprenyl) significantly decreased the neuroprotective effect of *l*-deprenyl (Magyar 1997), suggesting that a metabolite of *l*-deprenyl is an important part of that drug's neuroprotective effect. These studies, in combination with a study by Haberle and colleagues (2002), suggest that the effect of deprenyl may not be related to its MAO-B-inibitory activity. Haberle et al. (2002) found that rasagiline, which displays more potent inhibition of MAO-B than does *l*-deprenyl, did not prevent DSP-4-associated NA depletion. Rasagiline differs from *l*-deprenyl in that it is not metabolized to compounds that inhibit NA reuptake; neither is rasagiline, itself, an inhibitor of NA reuptake. Further, Finnegan and colleagues (1990) reported that a specific inhibitor of MAO-B, MDL-72974, failed to prevent DSP-4-induced NA depletion, further supporting the idea that MAO-B inhibition is not sufficient for prevention of DSP-4-associated neurotoxicity.

Despite this evidence in support of a role for NA reuptake, or at least a weak role for MAO-B inhibition in mediating protection against DSP-4 neurotoxicity, there also exists a significant body of data suggesting that MAO-B activity is sufficient and more important than inhibition of NA reuptake in preventing DSP-4-associated NA depletion. While some NA reuptake inhibitors, as discussed above, do protect against DSP-4-induced NA loss, others have failed in this regard. Clorgyline, a selective inhibitor of MAO-A, also inhibits NA reuptake but does not prevent DSP-4-induced NA depletion (Gibson, 1987). On the contrary, pargyline, a selective inhibitor of MAO-B and lacking an effect on inhibition of NA reuptake, does prevent NA depletion in this model (Gibson, 1987), suggesting a role for MAO-B inhibition rather than reuptake inhibition in neuroprotection in this model. The aliphatic propargylamine, *N*-2-hexyl-N-methyl-propargylamine (2-HxMP) is an inhibitor of MAO-B but not of NA reuptake and does prevent NA depletion caused by DSP-4 (Yu et al., 1994).

As part of the characterization of the neurochemical profiles of PEH and its analogues, these drugs were tested in the DSP-4 model and compared to *l*-deprenyl at a dose at which *l*-deprenyl has been reported to provide protection against the noradrenaline-depleting actions of DSP-4. The results of that screen are presented here.

6.2. Methods

6.2.1 Administration of DSP-4 and PLZ, PEH and PEH Analogues

The noradrenergic neurotoxin DSP-4 was prepared in physiological saline and injected at a dose of 50 mg/kg i.p. All drugs to be tested for neuroprotective activity were prepared in corn oil and injected at a dose equivalent, on a molar basis, to 10 mg/kg of *l*-deprenyl. All animals (male Sprague-Dawley rats) received two i.p. injections, the injections being separated in timing by 1 hour. Saline control animals received two injections of saline. Negative control animals received an initial injection of saline, with the second injection consisting of DSP-4 (50 mg/kg). Positive control animals received *l*-deprenyl as the initial injection and DSP-4 (50 mg/kg) as the second injection. All other animals received DSP-4 (50 mg/kg) as the second injection and one of either PLZ, PEH, FPEH, MePEH or MeOPEH as the first injection. Animals were returned to their cages following each injection. Following the second injection animals were allowed to survive, being monitored daily, for 7 days and were then killed by decapitation. After decapitation, hippocampi were rapidly dissected out of the animals, frozen in isopentane on solid carbon dioxide, then removed to other receptacles and stored at -80°C for later HPLC analysis.

6.2.2 HPLC Analysis of Rat Hippocampi for the Determination of Noradrenaline Content

Hippocampi were weighed and then homogenized in 5 volumes of ice-cold 0.1N perchloric acid containing EDTA (10 mg %) and ascorbic acid (50µM). The resultant homogenate was subjected to centrifugation (10,000 x g for 15 minutes) and the supernatants were put into HPLC vials for analysis. The column system consisted of a Waters µBondapak C18 pre-column coupled with a Spherisorb ODS 2 column (4.6 x 250 mm, 5 µm; Waters Corp., USA). The mobile phase consisted of 6.599 g of NaH_2PO_4, 197.2 mg sodium octyl sulfate, 137.7 mg EDTA, 80 mL CH_3CN and 0.117 g NaCl in 920 mL of distilled water, with pH adjusted to 2.9 using *o*-phosphoric acid and then filtered using a 0.2 µm filter. The mobile phase was used at a flow rate of 1.0 mL/minute. The column temperature was held at 30°C and analyte detection was performed by a Waters

2465 electrochemical detector (Waters Corp., USA). All samples were held at 4°C before injection and run time was approximately 30 minutes per sample. The only peak of interest in these experiments was that of NA, which was identified and quantified during analysis using Empower software for the PC. A calibration curve consisting of known, varying amounts of NA was run in parallel with each assay, and the amount of NA in the brain samples was calculated by extrapolation from the calibration curve.

6.3. Results

6.3.1 Effects of PLZ, PEH and PEH Analogues on DSP-4-Associated Noradrenaline Loss in Rat Hippocampus

Administration of PLZ, like that of *l*-deprenyl, resulted in a reduction in the NA-depleting effect of DSP-4 in rat hippocampi. Only the Vehicle-DSP-4 and FPEH-DSP-4 group produced a significant decrease in brain NA relative to the vehicle (Fig. 29). Although the PEH- and MePEH-treated groups did not have significantly lower NA than the vehicle-vehicle treated group, there was a trend towards a reduction in NA levels compared to the vehicle-vehicle treated group. However, when NA levels in animals treated with PEH or MePEH prior to DSP-4 were compared to those in the vehicle-DSP-4-treated rats, the NA levels, although showing a trend toward elevation, were not significantly elevated (Fig. 29). The experiment was repeated with FPEH, and again showed no protection against the DSP-4 depletion of NA at this dose of the drug.

6.4. Discussion

As described above, current explanations of the neuroprotective mechanisms of compounds such as *l*-deprenyl, rasagiline and clorgyline in the DSP-4 model have focused on the MAO-B-inhibitory activities of said compounds (in the case of *l*-deprenyl and rasagiline) and/or as their effectiveness as NA reuptake inhibitors, but controversy still remains about the relevance of these effects to the actions of drugs that are active in this neuroprotection screen.

The findings here with the potent MAO-B inhibitors PLZ and *l*-deprenyl and the PEH analogues, which are weak inhibitors of MAO, would appear to support the importance of inhibition of MAO-B in the DSP-4 model. Testing of PEH and its analogues as inhibitors of NA reuptake may be warranted, although such an action seems unlikely given the weak effect of PLZ on NA reuptake (McKenna et al., 1995). It is also of interest that PEH and its analogues have now been tested by other colleagues in the Neurochemical Research Unit at the 30 mg/kg dose used in the global ischemia studies and were found to be produce no neuroprotection.

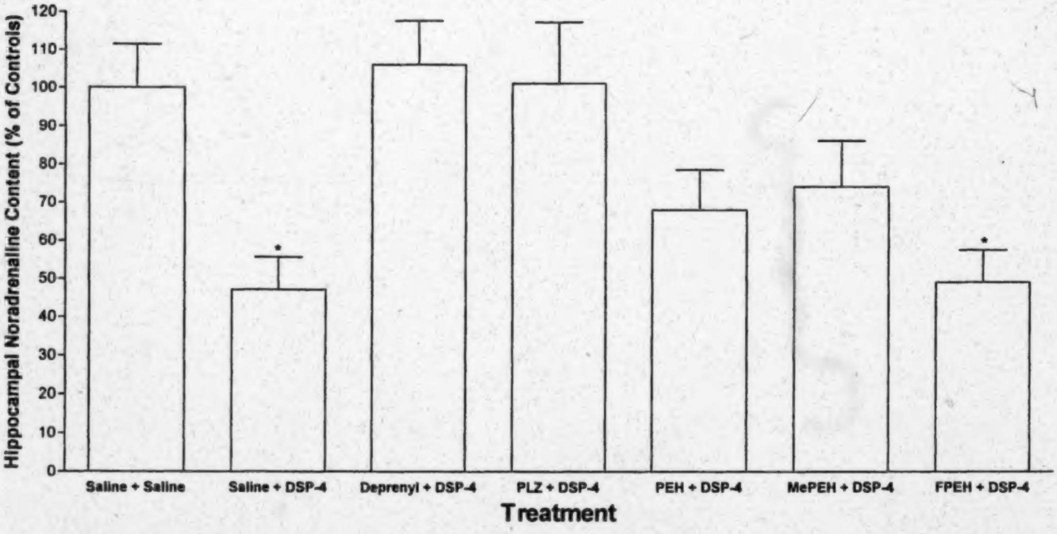

Figure 29. Effects of PLZ, PEH and PEH analogues on DSP-4-associated hippocampal noradrenaline loss in male Sprague Dawley rats, as determined by HPLC. Values are expressed as means ± SEM (N = 4). * indicates $p < 0.05$ vs Saline-Saline controls. Dunnet's Post test was used following ANOVA.

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Chapter 7

Hypothermic Effects of PEH Analogues

7.1. Introduction

Fever accompanies neurological symptoms in a significant proportion of stroke patients. Elevated core temperature has been linked with a poorer prognosis relative to normothermia in clinical and animal studies (Hindfelt, 1976; Zaremba, 2003 for review), while mild hypothermia has been associated with a better prognosis following stroke in human patients (Rieth and Jorgensen, 1996). In a retrospective study conducted by Hanchaiphiboolkul (2005) in which the medical records of 332 stroke patients who were admitted to hospital care within 48 hours of the onset of symptoms were examined, the highest recorded core temperature within the first 72 hours following admission was highly correlated with the risk of mortality. In rats in which strokes were experimentally induced via the injection of a blood clot into the middle cerebral artery, hyperthermia was associated with increased infarct size (Noor et al., 2003). In response to growing evidence implicating hyperthermia as a negative prognostic factor in stroke in both humans and experimental animals, attempts have been made towards preventing stroke-associated increases in body and brain temperature in order to improve survival and neurological outcome following stroke. Induced hypothermia has now been examined extensively in pre-clinical and clinical settings and has shown some promise.

Lowering of temperature has been shown experimentally to result in reductions in swelling (Kurokawa et al., 2001), intracranial pressure (Li et al., 1999; Feigin et al., 2002; Bernard and Buist, 2003), release of excitatory amino acids (Feigin et al., 2002; Bernard and Buist, 2003), metabolic rate and energy requirements (Bernard and Buist, 2003; Erecinska et al., 2003). The reduction in metabolic rate has allowed hypothermia to be used successfully in the preservation of tissues being used in transplantation operations (Erecinska et al, 2003). Reduction of cerebral temperature by 1 degree Celsius has been reported to in turn cause a 6-7% reduction in the cerebral metabolic rate (Bernard et al., 2003). As is further described by Erecinska et al. (2003), hypothermia has many effects on cerebral metabolism, including a reduction in energy synthesis (ATP metabolism), a reduction in energy utilization (ATP utilization, which actually decreases to a greater extent than does ATP production), reduced oxygen extraction from blood, increased oxygen saturation of blood and alkalinization of both intracellular and extracellular environments. In particular, hypothermia reduces the rate of flux of substrates through the glycolytic and tricarboxylic acid pathways, thereby reducing the utilization of glucose and the formation of lactate (Bernard and Buist, 2003); both of these effects would be beneficial in the setting of cerebral ischemia, where insufficient oxygen levels results in uncoupling of glycolysis from oxidative phorphorylation, increasing free radical formation. Consequent to the reduction in glycolysis (which is linked to lactate production in the setting of oxygen deficiency), the acid buildup that has been shown to accompany cerebral ischemia is reduced by hypothermia (Aoki et al., 1993; Chopp et al., 1989). Reduction in brain temperature results in a reduction of cerebral metabolic rate which, in the setting of cerebral ischemia should prove beneficial in light of the reduction in oxygen and glucose provision in the flow-compromised area.

There are now many reports of the use of hypothermia in pre-clinical studies on effecting neuroprotection following stroke in experimental animals. Interestingly, and fortunately, there are now both physical and pharmacologic means by which the lowering of body and brain temperature may be achieved.

While some experimental protocols have used water mist, cooling caps or cooling blankets to lower body temperature, more recent evidence suggests that drug-induced hypothermia may provide a reasonable and arguably more comfortable means by which a reduction in core and cerebral temperature may be achieved. Of particular interest is growing evidence that GABAergic agents are capable of inducing hypothermia. Hypothermic effects have been reported following administration of several GABAergic agents, including diazepam (Davies et al., 2004), tiagabine (Iqbal et al., 2002; Inglefield et al., 1995) muscimol, THIP, nipecotic acid, and GABA itself (Frosini et al., 2004). The hypothermia-inducing effects of tiagabine and diazepam were also demonstrated to contribute to their neuroprotective activity (Iqbal et al., 2002; Kuhmonen et al., 2002, Davies et al., 2004), and the temperature-lowering effect of diazepam has been demonstrated in humans as well as in experimental animals (Echizenya et al., 2003, 2004).

In light of the growing evidence that GABAergic agents are capable of inducing hypothermia both in experimental animals and in human subjects, PEH and its analogues were tested for their effects on gerbil core temperature following a single intraperitoneal injection. PLZ was also included in these experiments.

7.2. Methods

7.2.1 Examination of Drug Effects on Core Temperature

Gerbil core temperature was taken using a rectal thermometer prior to the animals being administered a single i.p. dose of vehicle (corn oil) or 30mg/kg drug (PLZ, PEH or PEH analogue). Rectal temperature measurements were repeated once per hour for 6 hours and then a final temperature measurement was made at 6.5 hours post-administration of either vehicle or drug. All rectal temperature measurements were performed while the animals were under halothane anesthesia. Between measurements, animals were permitted to roam in their cages with free access to food and water. In a separate set of experiments, the contribution of sleep-associated changes to the decrease in core temperature that was observed in drug-treated animals was examined by denying sleep to experimental subjects for the duration of the 6.5 hour experiment. Another set of experiments was performed to examine the effect of halothane anesthesia on core temperature over a 20 minute period, which represents the maximum anesthesia time for animals undergoing the surgical procedure described earlier (see Chapter 5). No effect of the anesthesia alone was found on core temperature.

7.3. Results

7.3.1 Time-Dependent Effects of PLZ, PEH and PEH Analogues on Core Temperature in Gerbils

FPEH, MePEH and MeOPEH all reduced core temperature by approximately 1°C, although the rapidity and duration of the reduction differed between drugs. Onset of hypothermia was first seen at 3 hours following administration of FPEH or MeOPEH, but at 2 hours post-administration in MePEH-treated animals (Fig. 30). The duration of the hypothermic episode was shortest with MeOPEH (2 hours) and longer with FPEH and MePEH (4 hours). These reductions in temperature were not as great in duration or extent or as fast in onset as those seen with PEH and PLZ (Fig. 30). Hypothermia was observed in gerbils one hour following administration of PEH, and normothermia had still not been reached even 7 hours post-administration (i.e. hypothermic time following PEH administration is greater than 6 hours). The maximum temperature reduction seen following PEH administration, 1.7°C, was also greater than those seen following administration of the PEH analogues (approximately 1°C). The reduction in temperature following administration of PLZ was even more striking, with core temperatures falling by an average of 4°C at 1 hour post-administration; as with PEH, animals remained hypothermic even 7 hours post drug-administration.

In animals forced to remain awake following administration of FPEH the hypothermia-inducing effect was still seen and the extent of the decrease in core

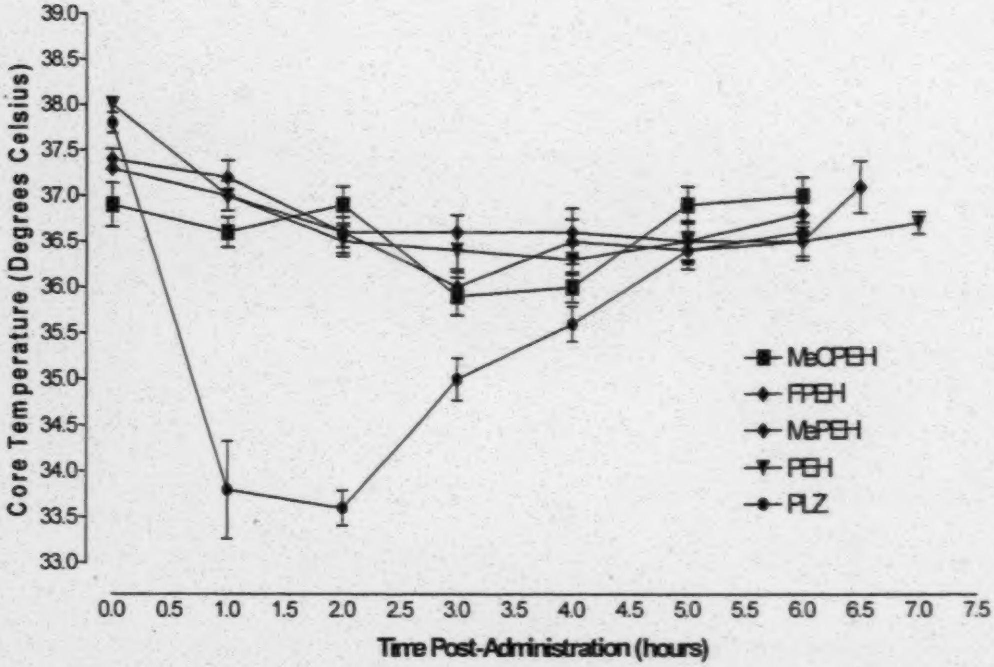

Figure 30. Time-dependent effects of PEH, PLZ and PEH analogues (30mg/kg, i.p. in each case) on core temperature. Data are expressed as means ± SEM (N = 6). $p < 0.05$ vs. controls as follows: 3 and 4 hours for MeOPEH; 3, 4, 5 and 6 hours for FPEH; 2, 3, 4 and 5 hours for MePEH; 1, 2, 3, 4, 5, 6 and 7 hours for PEH and PLZ.

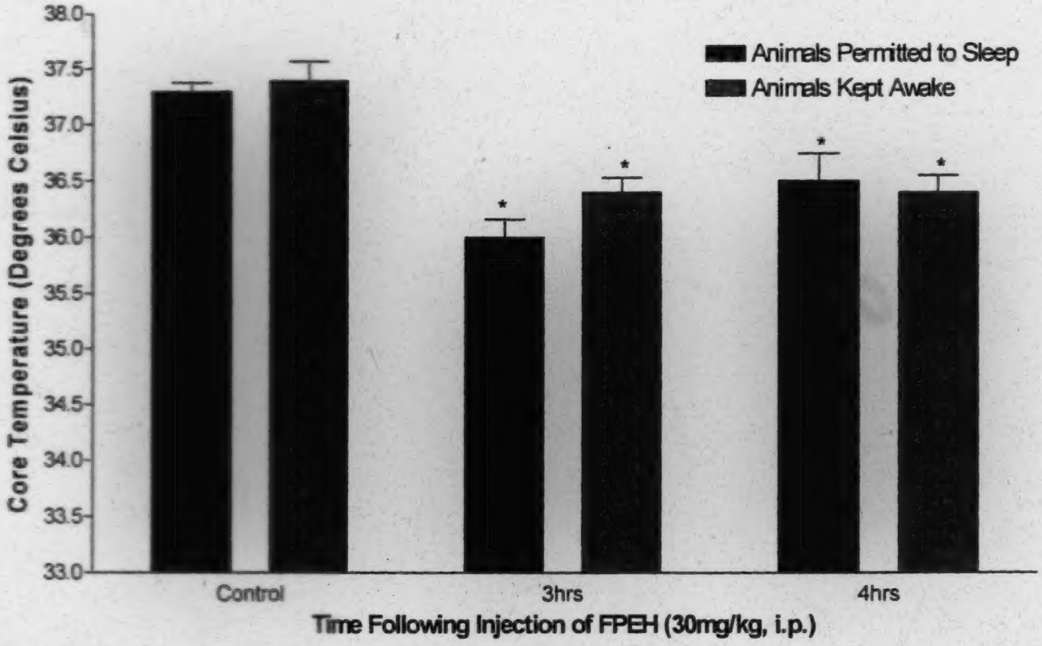

Figure 31. Contribution of sleep-associated reduction in core temperature to FPEH-associated changes in core temperature. Values represent means ± SEM (N=6). * indicates $p < 0.05$ vs. controls. $p > 0.05$ for a comparison of the two groups at 3 hours (permitted to sleep vs. denied sleep) and for the two groups at 4 hours (permitted to sleep vs. denied sleep).

temperature did not differ significantly from that seen in animals permitted to fall asleep following drug administration (Fig. 31).

7.4. Discussion

Studies with diazepam have shown it to augment GABAergic activity and induce hypothermia via direct effects at the level of the hypothalamus (Schwartz-Bloom and Sah, 2001). Additionally, a relationship between state of consciousness, specifically sleep, and body temperature has been described (Bach et al., 2002). The hypnotic effect of 30mg/kg doses of the PEH analogues necessitated examination of the contribution of changes in state of consciousness to the core temperature of animals to which sleep was denied for several hours following drug administration (Fig. 31). This examination revealed that, with FPEH, state of consciousness had no effect on animal core temperature. Temperature reduction of the same magnitude and duration was observed in animals to which sleep was denied following drug administration as in animals which were permitted to sleep following administration of FPEH; accordingly, it is likely that the effect of FPEH on core temperature is not related to the hypnotic effect of the drug, but rather effected by some other mechanism such as a direct effect at the level of the medial preoptico-anterior hypothalamus (mPOAH)(Jha et al., 2001) or the raphé pallidus (Zaretsky et al., 2003). These potential direct effects were not examined in this study and are suitable candidates for further examination. Which region, the raphé pallidus or the mPOAH, is more likely to be involved in the effect of GABAergic agents on core temperature is difficult to predict. As has been discussed, the effects of FPEH in this study appear to be independent of state of consciousness. Jha and colleagues (2001) demonstrated that picrotoxin-associated inhibition of GABAergic signaling in the mPOAH simultaneously affected state of consciousness and core temperature; however, those researchers did not attempt to dissociate the two effects and, accordingly, it cannot be said with certainty whether or not the hypothermic effect would have persisted had subjects been maintained awake in that study. Zaretsky et al. (2003) showed that injection of muscimol directly into this brain region resulted in a temperature reduction that was greatest approximately 1 – 1.25 hours post-injection. This time course is similar to that seen with PEH and its analogues, making this brain region particularly appealing so far as further examination of the relationship between PEH and its analogues and temperature regulation is concerned.

Differences in the depth and duration of hypothermia evoked by FPEH and MePEH do not appear sufficient to explain the greater neuroprotective activity of FPEH compared to MePEH described earlier in the gerbil ischemia model since slightly earlier onset of hypothermia and slightly longer duration of hypothermia occur with MePEH compared to FPEH. Interestingly, to my knowledge there are presently no data on the effect of repeated doses of GABAergic agents on body temperature – i.e. whether or not the body develops tolerance to the hypothermia-inducing effects of these drugs. Thus, this may prove to be a useful and interesting avenue for future investigation.

The marked hypothermic effect of PLZ is difficult to explain, but it might be reasonable to assume that it may be related to the elevation of brain monoamines such as 5-HT, NA and DA produced by this potent inhibitor of MAO and not evident with PEH and its analogues. This explanation is not entirely satisfactory, as some MAO inhibitors, including PLZ, have been reported to reverse the hypothermic effects of drugs such as morphine and reserpine (Botting et al., 1978; Milanes et al., 1987). However, the literature in this area is somewhat contradictory since the MAO inhibitor, moclobemide, has been demonstrated to induce hypothermia (Ulogol et al., 1995).

While up to this point only data in support of the use of hypothermia in stroke have been presented, it should also be noted that there are several caveats to the use of hypothermia as a clinical tool in the setting of cerebral ischemia. Specifically, in humans induction of hypothermia is inefficient and impractical, with cooling blankets providing slow induction of hypothermia that requires the use of general anesthetics and muscle relaxants to prevent shivering and reactive vasoconstriction (Bernard and Buist, 2003; Krieger and Yenari, 2004). This is in addition to the difficulty precisely controlling core temperature using a surface-cooling method. Induced hypothermia incurs additional risk of pneumonia as well as inducing diuresis, hypokalemia (due to a shift of potassium intracellularly) followed by hyperkalemia during re-warming, reduced phosphate concentrations, reduced numbers and function of white blood cells and platelets, and prolonged clotting times (Feigin et al., 2002; Bernard and Buist, 2003). Finally, thus far hypothermia has not been demonstrated to be effective in models of focal stroke when prolonged past 3 hours post-ischemia, although it was shown to be effective in a gerbil model global ischemia when given 6 hours post-ischemia (Colbourne et al., 2000; DeBow and Colbourne, 2003).

Physically induced hypothermia has thus far been shown to be an effective means, as effective as many pharmacologic interventions, towards effecting neuroprotection following stroke in some animal models of stroke. It has been shown to markedly reduce the behavioural deficits seen in gerbils in the open field test and T-maze following global ischemia (Colbourne et al., 1998) and to preserve electrical function of hippocampal CA1 neurons following cerebral ischemia (Dong et al., 2001). However, in light of the difficulties associated with physical cooling of experimental animals and human subjects, pharmacologically induced hypothermia such as that occurring following administration of PEH and its analogues represents a viable alternative and one worthy of future study in models of cerebral ischemia.

In summary, PLZ, PEH and the PEH analogues were all shown to reduce core temperature following administration of a single intraperitoneal dose to male Mongolian gerbils, although the effect was much more marked with PLZ than with PEH or the PEH analogues. While the mechanism by which this effect is elicited was not examined, previous reports in the literature implicate the mPOAH and the raphé pallidus in the hypothermia-inducing effects of GABAergic agents (Jah et al., 2001; Schwartz-Bloom and Sah, 2001; Zaretsky et al., 2003). The contributions of the hypothermic effects of PLZ, PEH and FPEH to their neuroprotective activities in the gerbil global ischemia model have not been determined experimentally at this point in time, and represent a reasonable future direction in which these studies could progress, as physically-induced hypothermia has been demonstrated to have a neuroprotective effect in preclinical stroke studies (Li et al., 1999; Colbourne et al., 2000; Dong et al., 2001; Kolmar et al., 2002; Colbourne et al., 2003) and to be associated with a better prognosis in human stroke patients (Jorgensen et al., 1999; Kollmar et al., 2002; Rieth and Jorgensen, 1996).

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Chapter 8

General Discussion and Future Directions

8.1 General Discussion

The PEH analogues examined in this thesis have shown themselves to resemble their parent compound, PEH, with respect to several neurochemical indices. The drugs all inhibit GABA-T and ala-T, causing elevation of brain GABA and alanine levels in acute studies. In chronic administration studies, all three PEH analogues caused an increase in frontal cortical levels of GABA in ischemic animals relative to ischemic animals treated with vehicle, while, surprisingly, alanine levels were similar in both groups. The reasons for the lack of effect on alanine in chronically treated ischemic gerbils are uncertain, but alanine is involved in a large number of metabolic pathways and it is possible that one or more of these may counteract the increase in alanine produced by inhibition of ala-T. For example, there is a direct metabolic relationship between alanine and lactate in the brain, and it is known that an increase in brain lactate is a common feature of cerebral ischemia (Aoki et al., 1993; Chopp et al., 1989). Such an increase in lactate may be partly the result of conversion of alanine to lactate (via pyruvate), reducing levels of alanine. As discussed in Chapter 5, the lack of alanine may have the effect of increasing glutamate catabolism and possibly reducing the coagonist effect of alanine on NMDA receptors.

FPEH displayed a particularly interesting effect on brain glutamate levels in both healthy and ischemic animals to which that drug was administered: FPEH caused not only an elevation of brain GABA levels, but a reduction in brain glutamate levels, after both acute and chronic administration. If, as has been suggested (Shuaib and Kanthan, 1997; Shuaib et al., 1997), the balance between GABA and glutamate is important in neuroprotection, the reduced glutamate in the presence of elevated GABA may account for the neuroprotective actions of FPEH relative to MePEH and MeOPEH in the gerbil global ischemia model. Both MePEH and MeOPEH also caused marked increases in GABA levels relative to those in ISCH+VEH gerbils, but only FPEH decreased glutamate levels significantly relative to the ISCH+VEH gerbils. It is also of interest that MePEH showed non-significant trends towards increased cell survival and decreased glutamate levels in this model; perhaps the neuroprotection would have been significant had the glutamate levels been significantly reduced in the ISCH+MePEH gerbils relative to the ISCH+VEH animals. Thus a study at a higher dose of MePEH may be warranted but, as indicated previously in this thesis, doses higher than 30 mg/kg cause considerable sedation.

As was mentioned in the introduction to this thesis, excessive extracellular glutamate such as occurs in cerebral ischemia results, through activation of various glutamate receptors, of a number of potentially pathological events. Antagonists of NMDA and AMPA receptors have been shown to have beneficial effects in animal models of stroke (Hassel and Dingeldine, 2006). It may be that the reduction of glutamate levels in the FPEH-treated animals may be contributing to the neuroprotective actions of this drug through decreased activation of glutamate receptors, and future studies of the effects of FPEH on those receptors could be of considerable interest.

As was mentioned earlier, administration of 7.5 mg/kg FPEH to healthy gerbils was associated with a significant reduction in brain glutamate, in addition to significant inhibition of GABA-T and ala-T (22% and 45%, respectively) but non-statistically significant changes in brain GABA and alanine levels. At 15 mg/kg, there was a reduction in glutamate levels accompanied by significant decreases in the activity of GABA-T and ala-T and a significant increase in brain GABA and alanine levels.

Our neurorescue experiments with gerbils have thus far only examined the effect of a 30 mg/kg dose of FPEH on neuronal survival following transient forebrain ischemia in gerbils. As is illustrated in Fig. 10, in healthy animals this dose was associated with the greatest GABA-T and ala-T inhibitory activity seen in the dose-response studies (35% and 60%, respectively), the greatest elevations of brain GABA and alanine levels seen in the dose-response studies (to 260 and 160 percent of controls, respectively) and only a 15% reduction in brain glutamate levels, which was not statistically significant. It is possible that a FPEH dose between 7.5 and 15 mg/kg would provide a good compromise between the GABA-elevating effects of the 15 mg/kg and 30 mg/kg doses of that drug combined with the glutamate lowering effect of the 7.5 mg/kg dose.

Ours is not the first report of a hill- or bell-shaped relationship between the use of a GABA transaminase inhibitor and a neuronal survival-related response. Costa et al. (2004) demonstrated, using cortical slices *in vitro*, a bell-shaped relationship between recovery of neuronal electrical activity following oxygen-glucose deprivation and dose of vigabatrin (irreversible inhibitor of GABA transaminase). In their study, GABA itself and the GABA transport inhibitor, tiagabine, also displayed bell-shaped relationships between their concentrations and the neuroprotective effects. Whether the bell-shaped relationships observed by Costa et al. (2004) are in any way related to the U-shaped relationship that we have observed between FPEH dose (and therefore GABA levels) and glutamate levels is not known.

Another potential component of the neuroprotective effect of FPEH is the mild hypothermia that was associated with administration of that drug. The contribution of the hypothermic effect to the neuroprotective effect of FPEH may be small since the FPEH-associated reduction in core temperature was similar to that seen with MePEH and MeOPEH, both of which were not neuroprotective. PLZ elicited the greatest reduction in core temperature of all of the compounds tested in this study, and previous work in the Neurochemical Research Unit showed that PLZ also elicited significant neuronal survival at doses lower than PEH (and FPEH in the study here) when administered post-ischemia to gerbils (Tanay et al., 2002). Since PLZ also inhibits MAO and elevates brain amines, it is possible that these effects contribute to the hypothermic effect of this drug, although, as mentioned in Chapter 7, the literature on the effects of MAO inhibitors on body temperature is contradictory. PEH, FPEH and MeOPEH at 30 mg/kg doses do not inhibit MAO-A or MAO-B sufficiently to cause an increase in brain levels of the biogenic amines NA, 5-HT or DA (Paslawski et al., 2001; Figs. 14 and 15; Sowa and Baker, unpublished). However, even if the MAO-inhibiting effect of PLZ is contributing to the hypothermia, it also carries the disadvantage, as mentioned earlier in this thesis, of possibly leading to potentially fatal interactions with tyramine-containing foods and with sympathomimetic amines; such interactions should not occur with PEH and its analogues.

The interaction of the PEH analogues with CYP isozymes warrants further investigation. The work outlined in this thesis did not attempt to ascertain whether the inhibition of CYP isozyme-mediated metabolism of specific substrates for those isozymes by PEH and its analogues represented inhibition or substrate competition for the active sites of the enzymes. It seems unlikely that they would be substrates since the most likely position of metabolism is hydroxylation at the 4 position of the phenyl ring, which is blocked by a substituent in FPEH, MePEh and MeOPEH. Irrespective of the actual nature of the interaction between these drugs and the CYP enzymes, the determination that PEH and its analogues inhibited CYP-mediated metabolism of other substrates is an important first step in ascertaining the pharmacokinetic profiles of these drugs, as it suggests that PEH and its analogues will likely also interfere with CYP isozyme-mediated metabolism of other drugs and xenobiotics that are normally subject to CYP-mediated metabolism. As was already mentioned in this thesis, many psychiatric drugs fall under this umbrella.

A weakness of the work in general on PEH and analogues to date has been the inability to relate changes in brain concentrations of these drugs or their possible metabolites to their neurochemical actions. Several members of the Neurochemical Research Unit have attempted to develop assays for PEH and its analogues using gas chromatography or HPLC, but difficulties in extracting the drugs, the presence of interfering peaks and instability of derivatives after extraction have hindered such efforts to date. The ability to measure levels of PEH and its analogues as well as their metabolites in future studies in discrete brain regions may help to explain some of the differences such as hypothermia and brain levels of glutamate, GABA and alanine observed among the analogues. Knowledge of the brain levels of the drugs will also allow us to ascertain if the *in vitro* findings with the various enzymes (GABA-T, ala-T and the CYP enzymes) are physiologically meaningful.

The neuroprotection studies undertaken in this thesis have dealt only with relatively short-term drug administration (i.e. experimental animals terminated at 7 days post-ischemia), but it is well established that neuronal survival at 7 days is not necessarily predictive of neuronal survival over longer durations (Schwartz-Bloom et al., 1998, 2000; Schwartz-Bloom and Sah, 2001). While our studies have established that FPEH is neuroprotective at 7 days post-stroke in the gerbil model of transient forebrain ischemia, FPEH has not, to date, been examined with respect to neuroprotective activity over a longer duration (28 or 35 days). Nor have such studies been conducted as yet with PLZ or PEH. Other GABAergic agents such as diazepam, tiagabine and imidazenil have been examined for the permanence of their neuroprotective effects (see Schwartz-Bloom and Sah, 2001 for review). Those studies showed a long-lasting neuroprotective effect of diazepam and revealed that tiagabine and imidazenil (partial agonist at benzodiazepine receptors) merely delay or slow cell death.

Longer term studies will now be conducted with PLZ, PEH and FPEH by another graduate student.

8.2 Other Possible Future Research

8.2.1 GABA$_B$ Receptor Effects/Contributions

Hypotheses regarding the potential neuroprotective effects of GABAergic agents, as well as examination of the neuroprotective effects of those agents, have focused on the GABA$_A$ receptor complex and its associated chloride channel. Post-synaptic GABA$_A$ receptors are involved in acute, phasic inhibitory responses. Presynaptic GABA$_B$ receptors are widely distributed in the CNS on neurons and glia (astrocytes and activated microglia) and are involved in more delayed and prolonged responses, mediated by modulation of K^+ and Ca^{2+} channel activity. While in healthy brain tissue the greatest role for GABA$_B$ receptors may involve feedback regulation of neurotransmitter release (and synthesis), this may not necessarily also be the case in ischemic brain tissue, and their role in the pathophysiology of neuronal death in ischemia may differ greatly from their role in healthy brain tissue.

Oscillation of the intracellular calcium concentration has been demonstrated in astrocytes in response to acetylcholine, glutamate, GABA and histamine (Charles et al., 2003). It is likely that neuronal activity (neurotransmitter release) is closely linked to astrocytic provision of metabolic precursors and modulation of cerebral blood flow (Zonta et al., 2003). Astrocytic GABA$_B$ receptor activation, resulting from GABA binding following release by inhibitory neurons, results in an increase in astrocytic intracellular Ca^{2+}

concentrations, astrocytic glutamate release and consequent feed-forward activation of inhibitory neurons (Charles et al., 2003). Activation of $GABA_B$ receptors has also been associated with increased activity of phospholipase-A_2 (PLA_2; Czuczwar and Patsalos, 2001), which catalyzes the liberation of arachidonic acid. Metabolism of arachidonic acid in the setting of ischemia may result in increased oxidative stress due to free radical formation. This is contrary to the inhibition, by $GABA_B$ receptor activation, of plasma membrane Ca^{2+} channels and positive modulation of plasma membrane K^+ channels in neurons (Schwartz-Bloom and Sah, 2001). The relative contributions of $GABA_A$ and $GABA_B$ receptors of neurons and glia must be determined, as must their relative contributions to cell death in the setting of cerebral ischemia.

8.2.2 Immunomodulatory Activities of PLZ, PEH and the PEH Analogues

It may be of considerable interest to investigate PLZ, PEH and analogues for immunomodulatory activities. GABAergic compounds have now been demonstrated to not only affect the function of neurons, but also of glial and immune cells. $GABA_A$ receptors on peripheral blood T cells have been implicated in the response of those cells to stimuli that would normally cause the proliferation of anti-CD3 and antigen-specific T cells *in vitro* (Tian et al., 1999). Activated microglial cells have been demonstrated to possess $GABA_B$ receptors that, upon activation, lead to the attenuation of interleukin-6 and interleukin-12 release, suggesting again a role for GABA and GABAergic drugs in modulation of immune system function (Kuhn et al., 2004). While the two examples described thus far speak to the effects of GABA on immune system function, the reciprocal relationship also exists. Interleukin-1β has been demonstrated, again *in vitro*, to inhibit $GABA_A$ receptor-associated chloride currents in primary hippocampal neurons (Wang et al., 2000).

8.2.3 In Vitro Examination of the Neuroprotective/Neurotoxic Effects of GABAergic Agents and $GABA_{A/B}$ Receptor Antagonists

Many studies, from basic science work to clinical trials, have been conducted using GABAergic agents based on the working hypothesis that potentiation/prolongation of GABAergic activity in ischemic brain tissue will result in neuroprotection due to the physiologic antagonism that exists between GABA and glutamate in the CNS of mature mammalian brain tissue (See Schwartz-Bloom and Sah, 2001 and Green et al., 2000 for reviews). While there have been several reports of the effects of GABA receptor activation on neuronal survival in *in vitro* models of cerebral ischemia (Costa et al, 2004; Kristensen et al., 2003; Nelson et al., 2001), Costa et al. (2004) are the first investigators to report on the neuroprotective activity of GABA, itself, in *in vitro* models of cerebral ischemia. They also presented evidence that vigabatrin, an irreversible inhibitor of GABA transaminase, possesses neuroprotective activity in an *in vitro* model of cerebral ischemia. Potential criticisms of the study, however, include: (1) presence of the putative neuroprotective drugs (tiagabine, vigabatrin or GABA) throughout the ischemic period, thereby creating a situation less relevant to the clinical reality of post-stroke drug administration than would have been the case had the drugs been present only during the latter stages of the ischemic period or had drug administration not been initiated until the reperfusion period began; (2) the short-term nature of the study (approximately 1-hr experiments examining neuronal electrical activity), which is likely necessitated by the short-lived nature of the experimental tissue; (3) the fact that the study does not address the question of the relative contributions of neurons vs astrocytes to the observed neuroprotective activity of the GABAergic drugs. With regard to the first criticism listed, it should be noted that relatively few *in vitro* studies have been conducted in which the putative neuroprotective agents were administered only during the simulated reperfusion period rather than prior to or during the simulated ischemic period. These types of studies are essential, especially given several observations: (1) the GABA-mimetic agent chlomethiazole, while neuroprotective in animal models of stroke, showed no neuroprotective activity in human stroke patients despite showing efficacy *in vitro* and *in vivo* using rodent models (Lyden et al., 2001); (2) metabolism and neuronal/astrocytic activity differ in severely ischemic vs moderately ischemic brain tissue (Haberg et al., 2001, Pascual et al., 1998); (3) GABA receptor activation, depending on the metabolic and developmental states of nervous tissue, can have both inhibitory and excitatory effects (Isomura et al., 2003; Chen et al., 1999; Schwartz-Bloom and Sah, 2001); (4) $\mathrm{GABA_A}$ and $\mathrm{GABA_B}$ receptors have different pharmacological profiles and distribution patterns, and may contribute differently to the pathophysiology of stroke. These observations suggest that there are still many holes in our current understanding of the pathophysiology of stroke; the studies alluded to earlier would address some of the deficiencies in the present state of knowledge of stroke and stroke pathophysiology, such that interventions can be more rationally developed.

While most systems for the examination of the responses of hippocampal neurons to *in vitro* ischemia have utilized immature neurons from neonatal rodents, Finley et al. (2004) have now demonstrated that adult hippocampal neurons are a viable system for the *in vitro* examination of neuronal survival and function. As mature and immature hippocampi likely differ in many respects relevant to the pathophysiology of stroke, the culture system developed by Finley et al. (2004) may prove more relevant to the study of adult human stroke (pathophysiology and potential interventions) than the current widely-used systems. It is well known, for example, that the chloride gradient in immature neurons differs from that seen in mature neurons (Schwartz-Bloom and Sah, 2001; Cupello, 2003). This difference is such that activation of GABA receptors in these cells can result in depolarization rather than hyperpolarization.

8.2.4 Neuron-Astrocyte Interactions

Astrocytes are involved in maintenance of the blood-brain barrier, regulation of extracellular ion concentrations (in the brain parenchyma), modulation of brain pH, and detoxification (modulation of ammonia levels). The GABA shunt pathway is important in several of these activities effected by a number of enzymes (GABA-T, GAD, SSADH, glutamine synthetase, glutaminase) that are present in neurons (GABA-T, GAD, SSADH, glutaminase) and astrocytes (glutamine synthetase) and are involved in the cycling of neurotransmitters, their metabolites and their precursors between astrocytes and neurons. Uptake of glutamate and, to a lesser extent, GABA by astrocytes allows those cells to participate in modulation of synaptic activity.

Astrocytes are also postulated to be involved in the provision of lactate to neurons during cerebral ischemia (Pellerin, 2003; Schurr et al., 1998), a scenario during which lactate serves as a substrate for neuronal oxidation in place of glucose. Important differences in the distribution of lactate dehydrogenase-1 (LDH1) and lactate dehydrogenase-5 (LDH5) have been reported, with LDH1 (primarily converts lactate to pyruvate) being found mainly in neurons and LDH5 (primarily converts pyruvate to lactate) being found mainly in astrocytes (Pellerin, 2003). Lactate has been demonstrated to preserve hippocampal slice function in the setting of glucose deprivation (Pellerin, 2003). In fact, Schurr et al. (1998) showed that, following hypoxia in hippocampal slices *in vitro*, lactate, rather than glucose, is an obligatory substrate for neuronal metabolism. Interestingly, astrocytes are capable of releasing lactate.

GABA uptake by astrocytes is not associated with a significant increase in intracellular Na^+ load and therefore does not trigger a large increase in ATP use by plasma membrane Na^+/K^+ ATPase enzymes (Chatton et al., 2003). This is contrary to activation of $GABA_B$ receptors by GABA, which is associated with a transient increase in intracellular calcium levels and subsequent activation of ATP-requiring Ca^{2+} pumps. Astrocytic glutamate uptake is also associated with increased Na^+/K^+ ATPase activity (Chatton et al., 2003).

8.2.5 Anticonvulsant Activity of PEH and its Analogues

Many GABAergic agents are presently in use in the treatment of epilepsy (Czuczwar and Patsalos, 2001; Frolund et al., 2002; Leker and Neufeld, 2003). Among these are two GABA-T inhibitors, namely valproic acid (VPA) and vigabatrin (VGB). While VPA is used in treating seizures of all types, VGB is only used as an adjunct in the treatment of intractable partial seizures with or without secondary generalization (Czuczwar and Patsalos, 2001). The limited clinical role of VGB is at least in part due to its demonstrated ophthalmic toxicity, consisting of visual field constriction, loss of visual acuity, defects in colour perception and retinal hyper- and hypo-pigmentation (Mejico et al., 2000). The ophthalmic effects are not a characteristic of GABAergic agents in general but are, in this class, unique to vigabatrin, which has been shown to accumulate in the retina, with retinal concentrations of vigabatrin reaching 5-times those seen in the brain (Sills et al., 2001).

Previous work in the Neurochemical Research Unit of the University of Alberta (Todd and Baker, unpublished) revealed PEH to be much more potent than vigabatrin in its GABA-T-inhibitory and GABA-elevating effects. In the context of demonstrated efficacy of GABAergic drugs in experimental assays of anticonvulsant activity and in clinical use in the treatment of epilepsy and seizure disorders, the observations made in the PEH studies mentioned above and the PEH analogue neurochemistry data presented in this thesis showing GABA-T-inhibitory activity and GABA-elevating effects of PEH analogues suggest that future investigation of PEH and its analogues for anticonvulsant activity is warranted. Phenelzine and PEH are presently being screened for antiepileptic activity in comparison to vigabatrin in collaborative studies between the Neurochemical Research Unit and other researchers.

8.3 References

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